Not enough can be enough: feasibility of the Idylla *EGFR* mutation test when reuse of stained tissue slides is the only option available

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ABSTRACT

Aims The minimally invasive procedures used in the diagnostic workup of patients with advanced non-small cell lung cancer (NSCLC) often provide poor yields of pathological material suitable for molecular analyses. Not infrequently, the DNA yield from small biopsies/cytological samples is insufficient for the assessment of genomic biomarkers that inform personalised therapies. The Idylla *EGFR* mutation test (IEMT) has been specifically designed to process formalin-fixed paraffin-embedded sections without requiring preliminary DNA extraction.

This study aims to evaluate the diagnostic accuracy of IEMT when used to analyse archival histopathology material. More specifically, our objective was to establish whether or not different staining procedures could affect assay performance.

Methods Twenty NSCLC samples were selected accordingly to *EGFR* mutational status. To mimic archived stained material, sections were subjected to H&E staining, fluorescent in situ hybridisation analyses or immunodetection by immunohistochemistry before being processed for IEMT.

Results Parallel assessment of *EGFR* mutational status by IEMT on stained sections and next-generation sequencing on DNA yielded a concordant result in 50 out of 60 tests (83.3%). The discoloration of H&E of the archived sample was found to be the optimal procedure to highlight all the actionable alterations of *EGFR*.

Conclusions IEMT can provide remarkable diagnostic accuracy for the assessment of *EGFR* mutational status also when the only source of pathological material available for molecular analyses is represented by H&E stained sections. Ad hoc supervision by a qualified molecular biologist is in any case recommended.

INTRODUCTION

Non-small cell lung cancer (NSCLC) is the predominant histotype among primary lung tumours.[1] Patients with NSCLC have a poor prognosis, with a 5-year overall survival of only 18%, mainly due to an advanced disease stage at diagnosis.[2]

Personalised therapies informed by the detection of actionable genomic biomarkers have radically modified the disease course in a considerable fraction of patients with NSCLC,[3] including those carrying *EGFR* mutations predictive of sensitivity to *EGFR* tyrosine kinase inhibitors (TKIs).[4 5]

International guidelines recommend next-generation sequencing (NGS) as the preferred methodology for this kind of analyses.[6] However, the implementation of NGS in the routine clinical practice is not always feasible, because many laboratories do not have the necessary expertise and sample workload required (cost/effectiveness). In addition, NGS has turnaround times that may cause potentially harmful delays in the clinical management of rapidly progressing patients.

A viable alternative to NGS for a rapid assessment of *EGFR* mutational status is the Idylla *EGFR* mutation test (IEMT for short, Biocartis NV, Mechelen, Belgium). IEMT is based on an approved (CE-IVD) fully automated real-time PCR—carried out in the Biocartis Idylla system—which detects *EGFR* mutations in formalin-fixed paraffin-embedded (FFPE) samples approximatively in a 150 min time.[7] The list of *EGFR* mutations detected by IEMT includes those in exons 18, 19, 20 and 21, that inform the clinical management of patients with NSCLC with *EGFR*-targeted drugs.[8 9]

Surgically resected tumours provide sufficient material for a range of molecular tests and therefore represent the best case-scenario for *EGFR* analyses.[10] Unfortunately, only a minority of patients with NSCLCs (nearly 30%) are eligible for surgical resection. For patients with advanced stage NSCLC, histopathological and molecular analyses must be performed on biopsies obtained through minimally invasive procedures, which may not provide sufficient material for the entire diagnostic workflow, including NGS.[11]

In these cases, optimisation of the available diagnostic material becomes an obligate choice, considering the frequent unfeasibility of a repeat biopsy. With this kind of scenario in mind, we endeavoured to assess the feasibility of assessing the *EGFR* mutational status using tissue sections already processed for the routine histopathology workflow. To mimic this condition, we selected 20 archival NSCLC samples with ascertained *EGFR* status, as previously determined by NGS, and carried out the Idylla assay on tissue slides already used for the routine histopathology diagnostic workflow (hematoxylin & eosin

(H&E) staining, immunohistochemistry (IHC) and fluorescent in situ hybridisation (FISH)).

MATERIAL AND METHODS

Study samples

Samples of primary or metastatic lung tumours obtained by different procedures (surgery, biopsy and fine needle aspiration) were selected retrospectively according to their *EGFR* status, as previously determined by NGS analyses with the Oncomine Solid Tumour DNA kit (CE-validated in vitro diagnostic) (Life Technologies), in order to analyse *EGFR* wild type (WT) and *EGFR*-mutated samples (exon 18, 19, 20 and 21).

All samples were handled in compliance with the current revision of the Declaration of Helsinki (Fortaleza, Brazil, 2013). All information regarding the human material was managed using anonymous numerical codes.

Sample preparation

In order to mimic a condition in which stained tissue slides are the only available source of material for molecular analyses, three to five sections were cut from FFPE tissue blocks and processed as follows. The first section was counterstained by H&E and was reviewed by the pathologist. Another tissue section was incubated with the anti-ALK monoclonal antibody clone D5F3 (Ventana Roche, Italy) and the immunoreaction was performed on a BenchMark XT Auto Stainer with the ultraView DAB detection kit (Ventana Medical Systems, Oro Valley, Arizona, USA). FISH analyses were carried out on yet another section using ALK probe 2p23.1-q23.2 (SPEC ALK Dual Colour Break Apart Probe, Zytovision) and, in order to quench the fluorescence signal, FISH slides were exposed to light for 24 hours (as documented in online supplemental figure 1A).

H&E and IHC tissue slides were quickly dehydrated in alcohol and mounted using CV MOUNT (Leica, Milan, Italy) after dipping in xylene. To dismount glass coverslips, slides were dipped in xylene and then rehydrated by exposing them to decreasing ethanol concentrations before water.

For FISH tests, we used DAPI as mounting medium; glass coverslips were applied onto slides in absence of glue. To perform Idylla assay, coverslips were removed manually.

This set of H&E/IHC/FISH slides was therefore used to carry out the Idylla test for each patient.

In all cases in which the concordance between NGS and IEMT data were incomplete, that is, at least one out of the three Idylla assays did not match the NGS data, IEMT was performed on two additional slides: one stained by H&E and subsequently discoloured (H&E Dis) through a 10 min incubation in citrate buffer (pH 6) at 98°C (as documented in online supplemental figure 1B), while the other one was directly processed as non-treated FFPE curl.

In order to evaluate whether diaminobenzidine (DAB) labelling could affect the Idylla assay, four slides obtained from surgically removed tumours and known to be TTF1 positive were selected. The samples were re-stained with the same TTF1 antibody (8G7G3 Agilent, Santa Clara, USA) using Bond Polymer Refine Detection on an automated autostainer (BondTM Max, Leica Biosystems, Milan, Italy). DAB was used as chromogenic substrate. After verifying TTF1 positivity and demounting procedure, these slides underwent the IEMT assay.

Idylla *EGFR* mutation test

The Idylla test is designed to detect 51 mutations in four *EGFR* exons: exon 18 (G719A/C/S), exon 19 (36 deletions), exon 20 (T790M, S768I and five insertions) and exon 21 (L858R and L861Q). To carry out the assay following the manufacturer's protocol, FFPE tissue curls are placed between two paper discs, directly into the cartridge of the fully automated Idylla instrument (Biocartis, Mechelen, Belgium) with a hands-on time of about 3 min. Inside the cartridge, a combination of chemical reagents, enzymes, heat and high-intensity focused ultrasound cause deparaffinisation, disruption of the tissue and cell lysis. In addition, the cartridge contains specific primers and fluorescent-labelled probes necessary for PCRs in five parallel multiplex chambers. The total turnaround time is 150 min. In parallel, for each chamber, a conserved fragment of the *EGFR* gene is also amplified as a PCR control (*EGFR* total) to monitor the reaction and the amount of amplifiable DNA. The Idylla system software converts fluorescence signals, generated by amplified DNA, into PCR curves and calculates, for each valid curve, a value of quantification cycle (Cq).

The presence of *EGFR* variant alleles is determined by the Idylla software through a ΔCq value obtained by the difference between the *EGFR* total Cq and the single mutation Cq. Samples with a ΔCq value outside a predefined range of assay validity are called as WT, that is, mutation 'not detected'.

Idylla system software elaborates and analyses amplicons and, by referring to a propietary decision tree, provides the specific mutation call. The manufacturer does not disclose the specific details of this decision tree as it is part of their intellectual property. In fact, Biocartis has yet to disclose the range in which the ΔCq values must fall to be considered positive and, therefore, is still unavailable for IEMT users. If no *EGFR* total signal is detected, the PCR is considered invalid. This condition may be due to insufficient DNA input, DNA fragmentation, presence of PCR reaction inhibitors or, in principle, cartridge-related problems.

The diagnostic output is provided as a report stating either 'no mutation detected' or '*EGFR* mutation detected', with a relative CQ (mean cycle of quantification) value, that is the mean value among the five *EGFR* total Cq reactions.

Idylla assay versus NGS

In the comparative analyses between NGS (considered as gold standard) and Idylla assay results, 'YES' corresponds to fully concordant results between the two methods, that is, all three IEMT assays matched NGS results in a given sample; 'PARTIAL' corresponds to a concordance between the two methods for at least one mutation in at least one of the three different sections; 'NO' corresponds to complete discordance between the two methods, that is, none of the IEMT assays in a given case matched NGS results.

In case of full concordance ('YES') among NGS and IEMT tests performed on three treated sections (H&E, IHC and FISH), no further investigation was made. For 'PARTIAL' and 'NO' cases, further analyses were carried out on two additional sections (H&E Dis and FFPE curl samples) and, eventually, on the archival DNA samples.

In the case of detected mutations, results were considered valid, regardless of the CQ value. Whenever mutations were not detected by IEMT, we were faced with two options: if CQ value was <24, no additional investigation was carried out, because, as stated in the manufacturer's instructions, a CQ<24 indicates a sufficient amount of input DNA and any issue of assay sensitivity can therefore be ruled out. If, in contrast, the CQ value was ≥24 and further analyses with a higher amount of input DNA (as suggested by the manufacturer) was not possible, PCR

Table 1 Histopathological characteristics of selected samples

Case	Histotype	Primitive or metastatic	Localisation	Source
1	Acinar ADC	Primitive	Lung	Surgery
2	Acinar, solid ADC	Primitive	Lung	Surgery
3	Acinar ADC	Primitive	Lung	Surgery
4	Acinar ADC	Metastatic	Lymph node	EUS FNAB
5	ADC	Metastatic	Pleura	Biopsy
6	Acinar, papillary ADC	Metastatic	Lymph node	EUS FNAB
7	Papillary ADC	Primitive	Lung	Surgery
8	Micropapillary, papillary ADC	Primitive	Lung	Surgery
9	Acinar, solid ADC	Primitive	Lung	Surgery
10	ADC	Metastatic	Lymph node	EUS FNAB
11	Solid ADC	Metastatic	Lymph node	Biopsy
12	Acinar ADC	Metastatic	Lymph node	EUS FNAB
13	ADC	Metastatic	Lymph node	EBUS TBNA
14	Mucinous, solid ADC	Primitive	Lung	Surgery
15	ADC	Metastatic	Pleura	Biopsy
16	ADC	Metastatic	Pleura	Biopsy
17	ADC	Primitive	Lung	Biopsy
18	ADC	Primitive	Lung	Surgery
19	ADC	Metastatic	Pleura	Biopsy
20	ADC	Primitive	Lung	Biopsy

ADC, adenocarcinoma; EBUS, endobronchial ultrasonography; EUS, endoscopic ultrasonography; FNAB, fine needle aspiration biopsy; TBNA, transbronchial needle aspiration.

Table 2 *EGFR* status detected by NGS analyses

Case	NGS						
	Tumour cells (%)	I mutation	Exon	AF (%)	II mutation	Exon	AF (%)
1	>70	G719C	18	42	S768I	20	39
2	90	E746_A750delELREA	19	82			
3	>70	E746_A750delELREA	19	25			
4	60	L858R	21	28	T790M	20	27
5	>70	E746_A750delELREA	19	8	S768I	20	8
6	30	WT					
7	>70	WT					
8	>70	E746_A750delELREA	19	18			
9	>70	L858R	21	26			
10	30	L858R	21	23			
11	50	L858R	21	30			
12	>70	L858R	21	39			
13	70	L858R	21	64			
14	70	E747_T751delLREAT	19	62	T790M	20	43
15	70	L747_P753>S	19	13			
16	60	L858R	21	53			
17	30	L858R	21	17			
18	>70	E746_T751>A	19	27	T790M	20	29
19	80	E746_A750delELREA	19	44			
20	20	L858R	21	16			

AF, allele frequency; NGS, next-generation sequencing; WT, wild type.

curve analyses were carried out, in order to evaluate the possible presence of amplified DNA below the pre-defined threshold of assay validity.

Statistical analyses

Results were evaluated through the application of the Cohen K statistic, which measures the agreement between two raters, and the relative 95% CIs were calculated. The qualitative interpretation of the results follows the Landis and Koch scale.[12]

RESULTS

Description of selected samples

We selected 20 advanced stage NSCLC samples (stage IIIb and IV). Histopathological characteristics of samples are summarised in table 1. By NGS analyses, 18 samples (90%) were *EGFR* mutated, as detailed in table 2. Overall, the selected samples contained eight different mutation types, with two co-occurring mutations being detected in five cases (25%) (table 2).

Idylla assay analyses

Table 3 indicates that concordance between Idylla and NGS analyses was found in 50 out of 60 tests (83%).

Specifically, H&E stained sections yielded two false negatives and one partial positive (ie, only one of two concomitant mutations was detected); IHC sections yielded one false negative and one partial; FISH sections yielded three false negatives and two partials. Of note, in all five cases with two co-occurring mutations at least one out of three tests identified both genomic variants.

Focusing on individual cases (table 3), 17 out of 20 cases (85%, (K=0.500; 95% CI: 0.05 to 0.95; p=0.010) were concordant when we looked at H&E stained sections. IHC stained sections were concordant in 18 out of 20 samples (90%, K=0.615; 95% CI: 0.15 to 1.00; p=0.003). Considering sections processed for FISH, NGS and Idylla assay results were concordant in 15 out of 20 samples (75%, K=0.342; 95% CI: 0.01 to 0.72; p=0.042).

The fact that IEMT tests on FISH samples were the least concordant, could indicate an interference of the FISH fluorophores with the fluorescence channels related to the mutations to be detected, as suggested by literature.[13]

Table 3 Agreement between NGS and Idylla assay results

Case	NGS		Idylla			Concordance
	I mutation	II mutation	H&E	IHC	FISH	
1	G719C	S768I	I+II	I+II	I+II	YES
2	E746_A750delELREA		I	I	I	YES
3	E746_A750delELREA		I	I	I	YES
4	L858R	T790M	I+II	I	I	PARTIAL
5	E746_A750delELREA	S768I	I+II	I+II	I	PARTIAL
6	WT		WT	WT	WT	YES
7	WT		WT	WT	WT	YES
8	E746_A750delELREA		I	I	I	YES
9	L858R		I	I	I	YES
10	L858R		I	I	I	YES
11	L858R		I	I	I	YES
12	L858R		I	I	I	YES
13	L858R		I	I	I	YES
14	E747_T751delLREAT	T790M	I+II	I+II	I+II	YES
15	L747_P753>S		WT	I	WT	PARTIAL
16	L858R		I	I	WT	PARTIAL
17	L858R		WT	WT	WT	NO
18	E746_T751>A	T790M	I	I+II	I+II	PARTIAL
19	E746_A750delELREA		I	I	I	YES
20	L858R		I	I	I	YES

FISH, fluorescence in situ hybridisation; H&E, hematoxylin & eosin; IHC, immunohistochemistry; NGS, next-generation sequencing; WT, wild type.

Table 4 Agreement between NGS and Idylla assay in TTF1 IHC positive cases

	NGS		Idylla	
Case	I mutation	II mutation	TTF1 IHC	Concordance
1	G719C	S768I	I+II	YES
2	E746_A750delELREA		I	YES
3	E746_A750delELREA		I	YES
7	WT		I	YES

IHC, immunohistochemistry; NGS, next-generation sequencing; WT, wild type.

In principle, DAB staining could affect performance of the Idylla assay. This possibility could not be tested using ALK-stained slides, because they were negative for ALK immunoreactivity. We therefore selected four surgically removed tumour samples which had been found to be TTF1 positive. Tissue slides were subjected to de novo anti-TTF1 IHC, which yielded strong positivity, as expected (table 4, see also online supplemental figure 2). IEMT performed from these slides yielded results fully concordant with NGS analysis (table 4), therefore ruling out that DAB-based IHC detection could interfere with the Idylla assay.

Looking at data reported in table 3, in 14 out of 20 cases (70%) we obtained the same result in the three independent IEMT tests. In the remaining six cases (reported as 'PARTIAL' or 'NO' in table 3), at least one of the tests yielded a result discordant from the NGS gold standard. Among these cases, case 17 was the only 'no case', that is, one in which IEMT failed to make the correct diagnosis, because all three tests yielded a false negative result.

We focused on discordant cases and explored alternative means to improve on IEMT diagnostic accuracy. To this end, IEMT assays were run on a fourth tissue slide which was stained with H&E and subsequently discoloured. The rationale here was that of eliminating the potential interference of the H&E stain on assay performance. In addition, we run IEMT on FFPE curls and controlled this new set of analyses by performing IEMT also with archival genomic DNAs previously used to carry out NGS tests. These data, reported in table 5, show that IEMT performed remarkably well on H&E Dis slides, yielding the correct diagnosis in six out of six cases. Most remarkably, this additional procedure allowed us to make the correct diagnosis for case 17, that is, the only 'no case' reported in table 3 (note that in this case we could not test archival DNA because this was not available). IEMT tests performed on FFPE curls were not as reliable as the H&E Dis tests, because they yielded an incorrect diagnosis in 50% of the cases. This was possibly caused by extended depletion of tumour cells in the fifth and last serial section.

Table 5 Additional comparative analyses on cases with discordant results between NGS and Idylla assay

	NGS		Idylla					
Case	Mutation	AF (%)	H&E CQ	IHC CQ	FISH CQ	H&E Dis CQ	FFPE curls CQ	DNA CQ
4	T790M	27	Yes 26.3	**No** 27.7	**No** 27.1	Yes 25.6	**No** 26.9	Yes 22.4
5	S768I	8	Yes 25.8	Yes 21.4	**No** 24.4	Yes 24.2	Yes 26.8	Yes 25
15	L747_P753>S	13	**No** 25.5	Yes 21	**No** 26	Yes 20	**No** 24.5	Yes 19.3
16	L858R	53	Yes 23.8	Yes 20.6	**No** 24	Yes 18.8	Yes 21.6	Yes 18.6
17	L858R	17	**No** 29.4	**No** 26.7	**No** 29.4	Yes 27.2	Yes 26.2	**Not available DNA**
18	T790M	29	**No** 25.2	Yes 22.2	Yes 21.1	Yes 22.1	**No** 24.3	Yes 18.8

AF, allele frequency; CQ, quality control; FFPE, formalin-fixed paraffin-embedded; FISH, fluorescence in situ hybridisation; H&E, hematoxylin & eosin; H&E Dis, hematoxylin & eosin discoloured; IHC, immunohistochemistry; NGS, next-generation sequencing.

Curves analyses of discordant cases

We noticed that in the 13 discordant tests, that is, those reported as 'No' in table 5, the CQ value was invariably ≥24. This contrasted with an optimal CQ value (<24) being observed in 64% (14/22) of the remaining cases, that is, those in which IEMT yielded a correct diagnosis. Given our assay conditions, we hypothesised that a CQ value ≥24 in the above tests might be caused primarily by an insufficient DNA input. If so, amplified DNA could be present in these IEMT assays, although at levels below the preset value, which the Idylla software requires for a confident mutation call. Therefore, we looked at DNA amplification profiles in the 13 discordant tests and found that a diagnostic curve of PCR products was present in four out of six cases, although at suboptimal levels. Representative Idylla PCR curves for variant reported as 'not detected' in table 5 are shown in figure 1, along with reference curves obtained in parallel assays run with archival control DNAs previously used for NGS.

In particular, cases 5 and 16 showed discordant results for *EGFR*-S768I and *EGFR*-L858R mutations, respectively, on the FISH sample (table 5).

Case 5 showed an *EGFR*-S768I amplification curve with a Cq value of 36.07; for case 16, the *EGFR*-L858R amplification curve showed a Cq value of 30.07 (figure 1A1,B1).

Case 15 showed discordant results for *EGFR*-L747_P753>S mutation, which is 'not detected' by Idylla assay on H&E, FISH and FFPE curl samples (table 5).

The C1 panel on figure 1 shows the real-time curve relative to *EGFR*-L747_P753>S mutation on FFPE curls (figure 1C1). The *EGFR*-L747_P753>S amplification curve has a Cq value of 24.5.

Case 18 showed discordant results for *EGFR*-T790M mutation on H&E and FFPE curls samples. The D1 panel on figure 1 shows the real-time curve obtained with Idylla assay on FFPE curls. The *EGFR* T790M amplification curve has a Cq value of 37.51.

DISCUSSION

The molecular diagnosis of mutations in *EGFR* exons 18, 19, 20 and 21 is recommended in all patients with advanced NSCLC, since TKI therapy provides significant improvement in survival and quality of life.[4 5] Most NSCLCs are diagnosed at an advanced disease stage, when surgery is not an option. Therefore, in more than 85% of cases, the clinical management of patients with NSCLC must rely on diagnostic procedures carried out on tissue samples obtained through minimally invasive procedures, that is, small biopsies or cytology sampling. Critically, both procedures may yield a poor amount of diagnostic material. Because H&E and IHC are prioritised in the routine diagnostic workflow, inadequate sampling may pose a serious challenge to the execution of NGS-based tests suitable for identifying genomic biomarkers that inform patients' assignment to molecularly targeted therapies.

With this in mind, we endeavoured to validate the use of the *EGFR* Idylla test in tissue slides already used for H&E, IHC and FISH analyses. In other words, we tested whether the Idylla test can be adapted to 'left over' diagnostic tissue slides whenever no other material is available for assessing *EGFR* mutational status. To this end, we tested whether H&E, IHC and FISH procedures

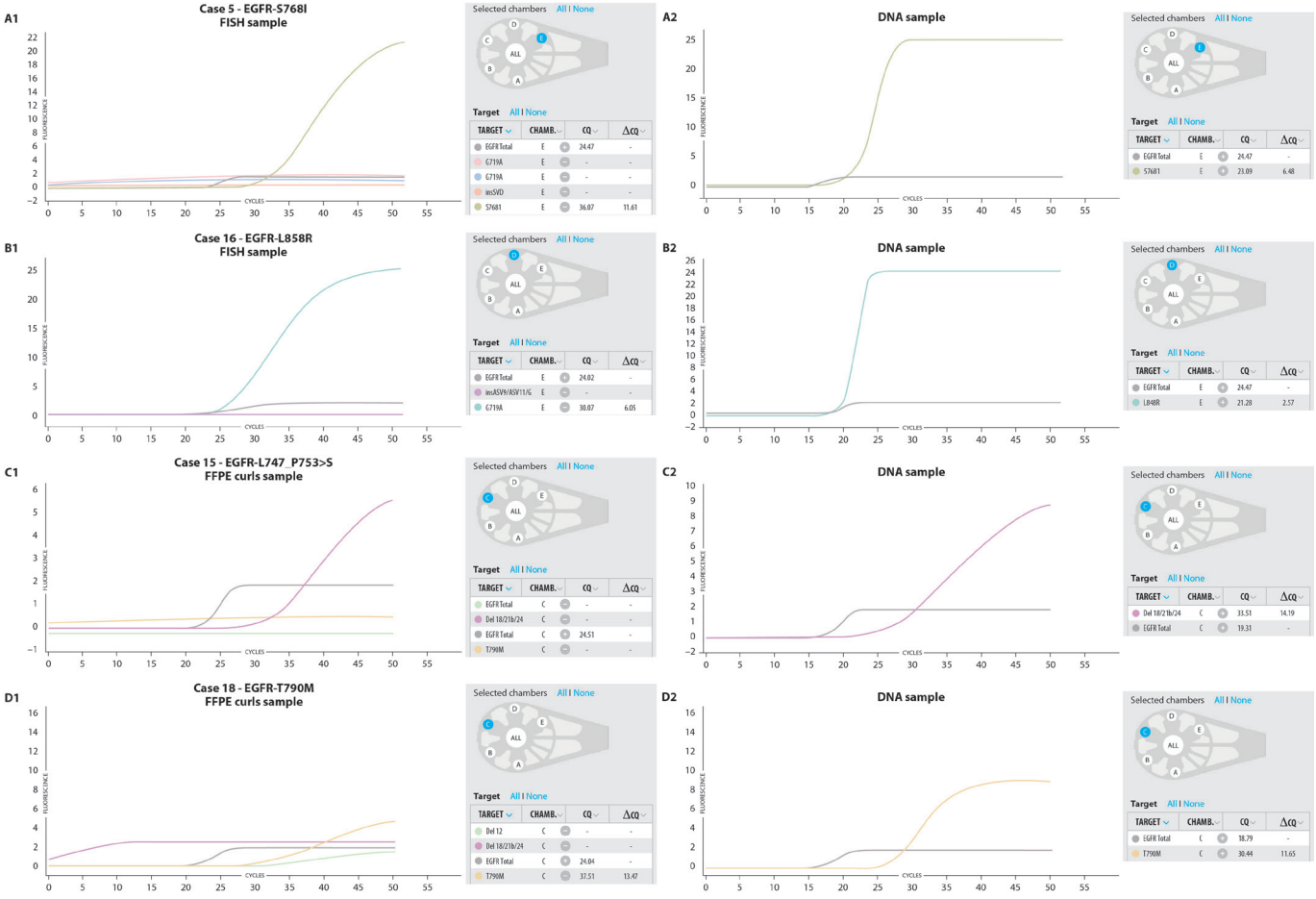

Figure 1 (A) Case 5. Real-time PCR analyses of the *EGFR*-S768I point mutation on FISH sample (1) and DNA sample (2). Green curve indicates the amplification of the S768I-mutant allele, black curve indicates the amplification of a conserved region of the *EGFR* gene and is used to assess the total amount of DNA sample. (B) Case 16. Real-time PCR analyses of the *EGFR*-L858R point mutation on FISH sample (1) and DNA sample (2). Blue curve indicates the amplification of the L858R-mutant allele, black curve indicates the amplification of a conserved region of the *EGFR* gene and is used to assess the total amount of DNA sample. (C) Case 15. Real-time PCR analysis of the *EGFR*-L747_P753>S point mutation on FFPE curls sample (1) and DNA sample (2). Pink curve indicates the amplification of the L747_P753>S-mutant allele, black curve indicates the amplification of a conserved region of the *EGFR* gene and is used to assess the total amount of DNA sample. (D) Case 18. Real-time PCR analysis of the *EGFR*-T790M point mutation on FFPE curls sample (1) and DNA sample (2). Yellow curve indicates the amplification of the T790M-mutant allele, black curve indicates the amplification of a conserved region of the *EGFR* gene and is used to assess the total amount of DNA sample. CQ, quality control; Cq, quantification cycle; FFPE, formalin-fixed paraffin-embedded; FISH, fluorescence in situ hybridisation.

interfere with IEMT analyses. Overall, our data demonstrate that the IEMT diagnostic pipeline applied to stained samples was concordant with NGS results in 50 out of 60 tests (83%) and allowed a correct assessment of *EGFR* mutational status in 19 out of 20 cases (having observed at least one of the three tests concordant with NGS). Remarkably, the three tests were uniformly concordant in the large majority of cases, that is, 14 out of 20 (70%). In the remaining 30% of cases, at least one of the three tests yielded a 'no mutation detected' result. These cases were further investigated, using H&E Dis and FFPE curls. The best results were obtained with H&E Dis samples, in which IEMT concordance with NGS was 100%. Most remarkably, H&E Dis allowed to make the correct diagnosis in case 17, namely the only case in which IEMT analyses on H&E, IHC and FISH slides had yielded a uniform 'no mutation detected' result. This datum suggests that H&E Dis offers the best conditions for IEMT analyses, possibly because H&E discolouration either increases the yield of amplified DNA or improves fluorescence signals detection. If this datum is confirmed in larger studies, IEMT testing on H&E Dis could represent the best option for *EGFR* mutational analyses in all instances in which poor yield of diagnostic material precludes NGS tests. Further support to the validity of our approach is provided by the consideration that the majority of samples analysed in the present study (60%, see table 1) were not obtained from surgically resected tumours. In these cases, archival FFPE tissue blocks had been already depauperated of the top three to four sections (ie, those previously cut and used for the routine diagnostic workflow). These conditions must be regarded as truly challenging, because, especially in the case of biopsies and endoscopic ultrasonography fine needle aspiration biopsy, the top three to four sections usually contain the highest share of tumour cells within the entire FFPE material.

We found that slides strongly positive to anti-TTF1 IHC (and therefore containing abundant DAB precipitates) could be reliably used in the Idylla assay. Thus, IHC positivity in DAB-based assays does not interfere with the Idylla assay. This finding implies that the use of IHC-processed tumour slides in the Idylla pipeline extends beyond ALK negative samples and may therefore include slides subjected to IHC procedures for differential diagnosis of lung cancer histotypes.

We noticed that in most of the discordant cases (four out of six, 66%) there was evidence of diagnostic DNA products, a datum strongly suggestive of suboptimal DNA input being responsible for incorrect diagnosis by IEMT. Thus, whenever CQ values of negative tests are ≥24 and no more tissue slides are available for further tests, it is important to examine PCR products curves and check whether suboptimal DNA amplification is present. We propose that the detection of suboptimal amounts of amplified DNA should alert about the still possible presence of an actionable *EGFR* mutation and therefore motivate further diagnostic attempts (eg, re-biopsy or liquid biopsy).

In detail, discordant results obtained only with FISH samples (cases 5 and 16) could indicate an interference of the FISH fluorophores with the fluorescence channels related to the mutations to be detected, as suggested by literature.[13]

Cases 15, 17 and 18 showed discordant results for *EGFR*-L747_P753>S, *EGFR*-L858R and *EGFR*-T790M mutations, respectively. A decrease in the number of CQ from normal H&E to H&E Dis has been observed on these samples. Consequently, the discordance is probably due to a channel interference of H&E staining, and the relative discolouration protocol allowed the Idylla system to detect the mutations. Discordant results relative to FFPE curls sample are probably caused by material depletion. In fact, for this study, FFPE curls samples were used as the last control because they are considered the gold standard of the Idylla assay method. However, being the last sections processed, the percentage of tumour cells was significantly lower compared with the other sections, leading to a failure of the assay in the 50% of cases.

Case 4 showed discordant results for *EGFR* T790M mutation which is 'not detected' by IEMT on IHC, FISH and FFPE curls samples, with a CQ value ≥24 in all these cases. Considering that the allele frequency (AF) of *EGFR* T790M variant found by NGS was 27%, probably Idylla assay failed to detect this missense mutation because of the low quality of the residual biological material reanalysed.

Within this study, the *EGFR* T790M mutation detected by NGS below the diagnostic cut-off value of AF 5% (data not shown) has been verified. To this end, two cases (16 and 17) with the *EGFR* T790M mutation detected by NGS with an AF <5% have been selected and analysed with Idylla assay. Such mutations were not detected by the IEMT in both samples, also in the archival DNA previously used for NGS, confirming the negativity for this mutation. Consequently, it can be assumed that the T790M mutation detected on DNA by NGS with a low AF, could represent a formalin fixation artefact.[14]

Take home messages

- ⇒ The Idylla assay allows reliable detection of actionable *EGFR* mutations in formalin-fixed paraffin-embedded tissue slides already used for the routine pathology workflow in non-small cell lung cancer.
- ⇒ Discoloured H&E sections provided the best results.
- ⇒ To guarantee the execution of the test, in case of limited pathological material, two consecutive H&E sections should be prepared, while also allowing that an H&E section is archived.
- ⇒ It is necessary and mandatory that Idylla *EGFR* mutation test be used by expert biologists able to interpret even the raw data, for a correct results interpretation.

The deep analyses on discordant samples, in particular for real-time curves, suggest that although the IEMT is a fast and precise system in identifying *EGFR* mutations, it is necessary and mandatory that it be used by expert biologists able to interpret even the raw data, for a correct results interpretation.

Moreover, we have shown that discoloured H&E sections provided the best Idylla *EGFR* results. Thus, we would like to propose that, whenever limited availability of pathological material is an issue, two consecutive H&E sections should be prepared, in order to guarantee the execution of the *EGFR* mutational analyses, while also allowing that an H&E section is archived.

Alternatively, laboratories that have already introduced the digital pathology methods and therefore have a digital archive of H&E slides at their disposal could, through discolouration techniques, use the original H&E slide directly.

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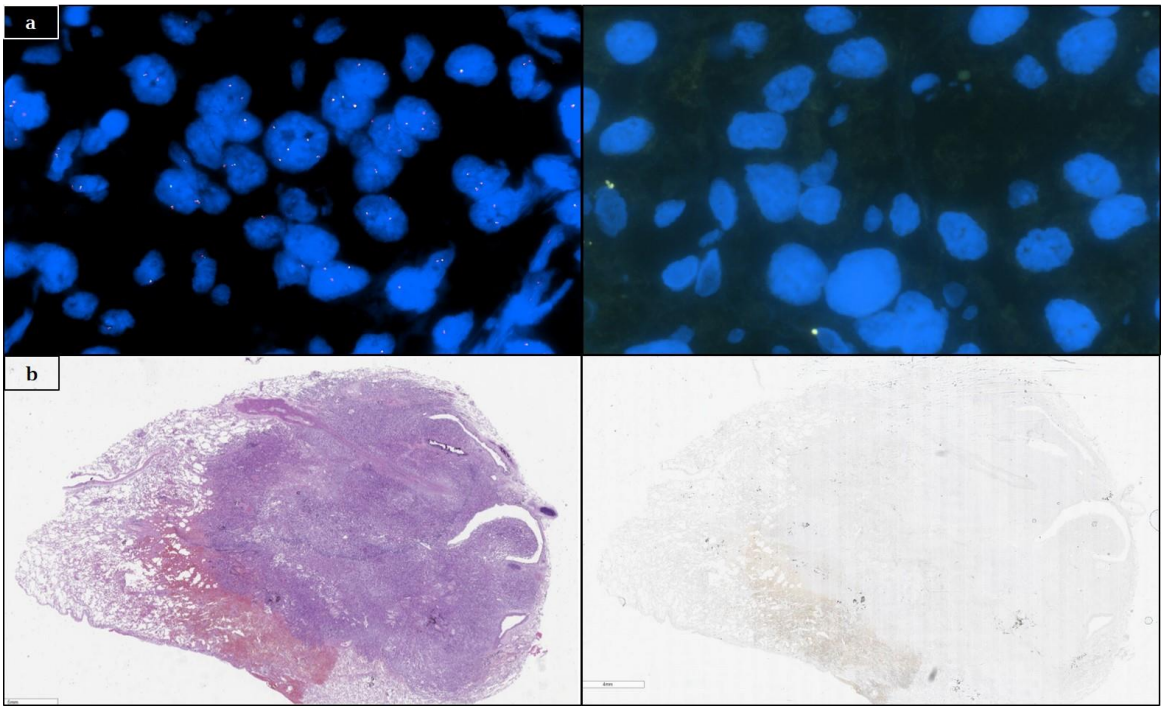
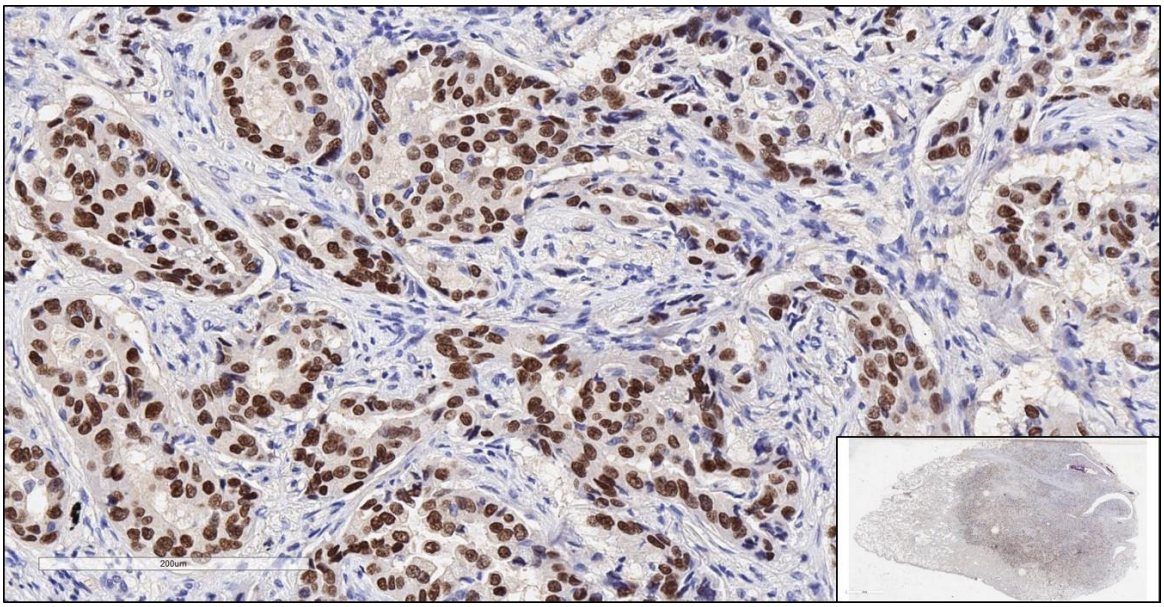
Supplemental Figure 1



Supplemental Figure 1. A representative sample of NSCLC (case 1): **a)** ALK FISH before (left) and after (right) exposure to light for 24 hours. **b)** HE staining before (left) and after (right) discoloration procedure. Scale bar 4 mm.

Supplemental Figure 2



Supplemental Figure 2. A representative sample of NSCLC (case 1) showing a nuclear TTF1 strong positivity. Scale bar 200 µm.