The Journal of Molecular Diagnostics, Vol. 21, No. 6, November 2019

ELSEVIER

the Journal of Molecular Diagnostics

jmd.amjpathol.org

Multicenter Evaluation of the Fully Automated PCR-Based Idylla EGFR Mutation Assay on Formalin-Fixed, Paraffin-Embedded Tissue of Human Lung Cancer

Solène M. Evrard,[*,†] Estelle Taranchon-Clermont,[*] Isabelle Rouquette,[*] Samuel Murray,[‡,§] Sebastian Dintner,[¶] Yun-Chung Nam-Apostolopoulos,[¶] Beatriz Bellosillo,[||] Mar Varela-Rodriguez,[**] Ernest Nadal,[††,‡‡] Klaus H. Wiedorn,[§§] Linea Melchior,[¶¶] Emma Andrew,[||||] Mary Jones,[||||] Jennifer Ridgway,[***] Christina Frykman,[†††] Linda Lind,[†††] Mitja Rot,[‡‡‡] Izidor Kern,[‡‡‡] Ernst J.M. Speel,[§§§] Guido M.J.M. Roemen,[§§§] Nicol Trincheri,[¶¶¶] Sandra N. Freiberger,[||||||] and Markus Rechsteiner[||||||]

From the Pathology and Cytology Department,[*] Toulouse University Hospital (CHU), Toulouse Cancer University Institute (IUCT) Oncopole, Toulouse, France; the Histology and Embryology Department,[†] Rangueil Medical School, Toulouse III Paul Sabatier University, Toulouse, France; BioPath Innovations S.A.,[‡] Athens, Greece; BioMarker Solutions Limited,[§] London, United Kingdom; the Institute of Pathology,[¶] Klinikum Augsburg, Augsburg, Germany; the Laboratori de Diagnòstic Molecular,[||] Servei de Patologia, Hospital del Mar, Barcelona, Spain; the Pathology Department,[**] Hospital Universitari de Bellvitge, Hospitalet, Barcelona, Spain; the Department of Medical Oncology,[††] Catalan Institute of Oncology, Hospitalet, Barcelona, Spain; the Clinical Research in Solid Tumors (CReST) Group,[‡‡] Oncobell Program, The Bellvitge Institute for Biomedical Research (IDIBELL), L'Hospitalet, Barcelona, Spain; the Molecular Pathology Department,[§§] Medizinisches Versorgungszentrum (MVZ) Zentrum für Pathologie und Zytodiagnostik GmbH (ZPZ) Köln, Cologne, Germany; the Department of Pathology,[¶¶] Rigshospitalet, Copenhagen, Denmark; the Molecular Cell Pathology Unit,[||||] Histopathology, Royal Cornwall Hospital, Cornwall, United Kingdom; the Molecular Pathology Department,[***] ACT Pathology, Canberra Hospital, Garran, Australian Capital Territory, Australia; the Clinical Pathology and Cytology Department,[†††] Laboratoriemedicin Gävle, Gävleborg, Sweden; the Laboratory for Cytology and Pathology,[‡‡‡] University Clinic for Respiratory and Allergic Diseases Golnik, Golnik, Slovenia; the Department of Pathology,[§§§] GROW—School for Oncology and Developmental Biology Department, Maastricht UMC+, Maastricht, the Netherlands; the Department of Pathology,[¶¶¶] Humanitas Research Hospital, Milan, Italy; and the Department of Pathology and Molecular Pathology,[||||||] Diagnostic Tumor Genome Analysis University Hospital Zurich, Zurich, Switzerland

Accepted for publication June 13, 2019.

Address correspondence to Solène M. Evrard, Pharm.D., Ph.D., Pathology and Cytology Department, CHU Toulouse, IUCT Oncopole, 1 avenue Irène Joliot Curie, 31059 Toulouse Cedex 9, France. E-mail: solene.evrard@univ-tlse3.fr.

Before initiating treatment of advanced non—small-cell lung cancer with tyrosine kinase inhibitors (eg, erlotinib, gefitinib, osimertinib, and afatinib), which inhibit the catalytic activity of epidermal growth factor receptor (EGFR), clinical guidelines require determining the EGFR mutational status for activating (*EGFR* exons 18, 19, 20, or 21) and resistance (*EGFR* exon 20) mutations. The EGFR resistance mutation T790M should be monitored at cancer progression. The Idylla EGFR Mutation Assay, performed on the Idylla molecular diagnostics platform, is a fully automated (<2.5 hours turnaround time) sample-to-result molecular test to qualitatively detect 51 *EGFR* oncogene point mutations, deletions, or insertions. In a 15-center evaluation, Idylla results on 449 archived formalin-fixed, paraffin-embedded tissue sections, originating from non—small-cell lung cancer biopsies and resection specimens, were compared with data obtained earlier with routine reference methods, including next-generation sequencing, Sanger sequencing, pyrosequencing, mass spectrometry, and PCR-based assays. When results were discordant, a third method of analysis was performed, when possible, to confirm test results. After confirmation testing and excluding invalids/errors and discordant results by design, a concordance of 97.6% was obtained between Idylla and routine test results. Even with <10 mm^2 of tissue area, a valid Idylla result was obtained in 98.9% of the cases. The Idylla EGFR Mutation Assay enables sensitive detection of most relevant *EGFR* mutations in concordance with current guidelines, with minimal molecular expertise or infrastructure. *(J Mol Diagn 2019, 21: 1010—1024; https://doi.org/10.1016/j.jmoldx.2019.06.010)*

Supported by Departament de Salut de la Generalitat de Catalunya—PERIS (Health Research and Innovation Strategic Plan) Program SLT006/17/00127 (E.N.) and the Department of Pathology of Copenhagen (L.M.).

Disclosures: I.R. has worked as consultant and participated in advisory boards from Astra-Zeneca, BMS, Boehringer, MSD, Roche, and Takeda; B.B. has patent royalties from Biocartis and honoraria for speaker consultancy or advisory role from Astra-Zeneca, Biocartis, Merck-Serono, Novartis, Qiagen, Hoffman-La Roche, and ThermoFisher; E.J.M.S. has received a research grant from AstraZeneca.

Copyright © 2019 American Society for Investigative Pathology and the Association for Molecular Pathology. Published by Elsevier Inc.
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0).
https://doi.org/10.1016/j.jmoldx.2019.06.010

Lung cancer is one of the four most common cancers in Europe and the United States and is undoubtedly one of the deadliest.[1−3] Smoking is the primary risk factor for lung cancer.[4] Lung cancer is composed of two main histologic subtypes: non−small-cell lung cancer (NSCLC) and small-cell lung cancer, with NSCLC being the most frequently diagnosed subtype (ie, 85%).

Prognosis is poor after advanced NSCLC diagnosis, with the vast majority of patients not surviving, despite treatment.[5] An important improvement in the treatment of metastatic NSCLC was the development of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), such as afatinib, erlotinib, and gefitinib.[6,7] Those TKIs inhibit the catalytic activity of mutated EGFR, a member of the tyrosine kinase family comprising several isoforms.[8,9] Although TKIs improve overall and progression-free survival, and overall response in NSCLC, resistance to this class of compounds usually develops within 9 to 12 months after initiation of therapy.[10]

EGFR is the gene that is most frequently mutated in NSCLC in the Asian population (ie, in approximately 50% of the Asian patients) and often in the Western population (ie, in approximately 10% of patients), especially in never smokers.[11] At baseline, exon 19 deletions and point mutation L858R in exon 21 account for approximately 85% to 90% of all *EGFR*-activating mutations.[12] Less prevalent EGFR mutations, in particular G719A/C/S, T790M, S768I, exon 20 insertions, and L861Q, constitute the remaining 10%.[13] The presence of most of these EGFR mutations is predictive for sensitivity to TKIs,[14] leading to improved progression-free survival but without demonstrable impact on overall survival; they are accordingly referred to as sensitizing or activating EGFR mutations.[15,16] An exon 20 insertion, on the contrary, may predict resistance.[17] Hence, it is required by guidelines to determine the EGFR mutational status in advanced NSCLC for activating or resistance mutations before initiation of TKI therapy.[18−21] Appearance of point mutation T790M in exon 20 of *EGFR* is pivotal in the development of resistance to TKIs[10]; and, therefore, T790M should be monitored at least at disease progression. Third-generation TKIs, like osimertinib, have been designed to target the T790M mutation and are used for treatment of patients who have developed this resistance mutation.[18,22] Recently, osimertinib has also been approved by the US Food and Drug Administration as a first-line treatment for both sensitizing and T790M-mutated tumors.[23,24]

Routinely, EGFR mutation analysis is performed on tumor tissue acquired by surgery or biopsy.[25] However, tumor tissues are not always satisfactory accessible, and repeated surgery or biopsy to assess treatment resistance is often inappropriate. Moreover, surgery and biopsy might cause clinical complications, with intrathoracic biopsies having a complication rate of 17.1%.[26] As a result, tissue samples are often small and may have limited tumor content.

Several commercially available CE-IVD kits and in-house assays, based on Sanger sequencing, pyrosequencing, next-generation sequencing (NGS), real-time PCR, or mass spectrometry, have been developed to routinely assess the mutational status of formalin-fixed, paraffin-embedded (FFPE) NSCLC tissue samples.[27,28] Each of these methods has its sensitivity, specificity, level of automation and multiplexing, turnaround time, cost, and requirement for specialized equipment and trained staff.

The Idylla EGFR Mutation Assay (Biocartis, Mechelen, Belgium), performed on the Idylla platform, detects 51 EGFR mutations in FFPE NSCLC tissue samples. Biocartis has since launched a CE-marked IVD (Idylla EGFR Mutation Test) intended to determine the tumor EGFR mutation status of patients with metastatic NSCLC at diagnosis of advanced disease and to facilitate treatment decisions within a multidisciplinary team. The Idylla EGFR Mutation Test is the only fully automated CE-IVD test detecting the mutations in *EGFR* exons 18, 19, 20, and 21 that are considered clinically relevant, according to current international guidelines.[18−21]

In the current study, a multicenter evaluation (15 centers) of the performance of the Idylla EGFR Mutation Assay on 449 archived FFPE tissue sections, originating from NSCLC lesions, in comparison with data retrospectively obtained with routine reference methods on matched samples is described.

Materials and Methods

Multicenter Study Design

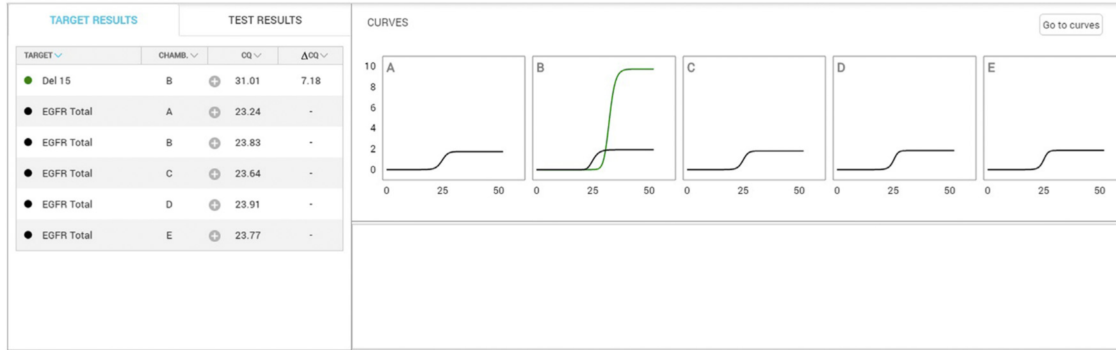
Archived clinical FFPE tissues of 449 NSCLC patients were selected for this study. Samples were obtained from 15 clinical centers: BioPath Innovations S.A./BioMarker Solutions Limited (Athens, Greece; London, UK; $n = 28$); Klinikum Augsburg (Augsburg, Germany; $n = 29$); Hospital del Mar (Barcelona, Spain; $n = 30$); Hospital Universitari de Bellvitge/Catalan Institute of Oncology (Hospitalet, Spain; $n = 30$); University of Coimbra (Coimbra, Portugal; $n = 31$); Medizinisches Versorgungszentrum (MVZ) Zentrum für Pathologie und Zytodiagnostik GmbH (ZPZ) Köln (Cologne, Germany; $n = 31$); Rigshospitalet (Copenhagen, Denmark; $n = 30$); Royal Cornwall Hospital (Cornwall, UK; $n = 29$); Canberra Hospital (Garran, ACT, Australia; $n = 33$); Laboratoriemedicin Gävle (Gävleborg, Sweden; $n = 28$); University Clinic for Respiratory and Allergic Diseases (Golnik, Slovenia; $n = 30$); Maastricht UMC+ (Maastricht, the Netherlands; $n = 30$); Humanitas Research Hospital (Milan, Italy; $n = 30$); Toulouse Cancer University Institute (IUCT) Oncopole (Toulouse, France; $n = 30$); and University Hospital Zurich (Zurich, Switzerland; $n = 30$). The use of these patient samples was approved by the respective local Ethics Committees and was in accordance with the Declaration of Helsinki. Each of the 15 participating clinical centers was asked to select and include at least one NSCLC

FFPE sample for each of the seven Idylla EGFR Mutation Assay genotype calls indicated in Table 1.

In this study, Idylla EGFR Mutation Assay cartridges, processed by the Idylla System, were used to analyze the EGFR mutational status of these archival FFPE tissue sections from human NSCLC tissue. Participating centers received proper training to perform the Idylla EGFR Mutation Assay before starting the study. The FFPE tissue

Table 1 *EGFR* Mutations Detected by the Idylla EGFR Mutation Assay

Exon	Mutation	Protein change	Nucleotide change	Genotype call Idylla
18	G719A	p.Gly719Ala	c.2156G>C	G719A/C/S
	G719C	p.Gly719Cys	c.2155G>T	
			c.2154_2155delinsTT	
	G719S	p.Gly719Ser	c.2155G>A	
19	Del 9	p.Leu747_Ala750delinsPro	c.2238_2248delinsGC	Exon 19 deletion
			c.2239_2248delinsC	
		p.Leu747_Ala750delinsSer	c.2240_2248del	
		p.Leu747_Glu749del	c.2239_2247del	
	Del 12	p.Leu747_Thr751delinsPro	c.2239_2251delinsC	
		p.Leu747_Thr751delinsSer	c.2240_2251del	
	Del 15	p.Glu746_Ala750del	c.2235_2249del	
			c.2236_2250del	
		p.Leu747_Thr751del	c.2239_2253del	
			c.2240_2254del	
			c.2238_2252del	
		p.Glu746_Thr751delinsAla	c.2237_2251del	
		p.Glu746_Thr751delinsIle	c.2235_2252delinsAAT	
		p.Glu746_Thr751delinsVal	c.2237_2252delinsT	
		p.Lys745_Ala750delinsThr	c.2234_2248del	
		p.Glu746_Thr751delinsLeu	c.2236_2253delinsCTA	
		p.Glu746_Thr751delinsVal	c.2237_2253delinsTA	
		p.Glu746_Thr751delinsAla	c.2235_2251delinsAG	
		p.Glu746_Thr751delinsGln	c.2236_2253delinsCAA	
		p.Ile744_Ala750delinsValLys	c.2230_2249delinsGTCAA	
	Del 18	p.Leu747_Pro753delinsSer	c.2240_2257del	
		p.Glu746_Ser752delinsVal	c.2237_2255delinsT	
		p.Leu747_Ser752del	c.2239_2256del	
		p.Glu746_Thr751del	c.2236_2253del	
		p.Leu747_Pro753delinsGln	c.2239_2258delinsCA	
		p.Glu746_Ser752delinsAla	c.2237_2254del	
		p.Glu746_Ser752delinsAsp	c.2238_2255del	
		p.Glu746_Pro753delinsValSer	c.2237_2257delinsTCT	
		p.Glu746_Ser752delinsIle	c.2236_2255delinsAT	
			c.2236_2256delinsATC	
		p.Glu746_Ser752delinsVal	c.2237_2256delinsTT	
			c.2237_2256delinsTC	
			c.2235_2255delinsGGT	
	Del 21	p.Leu747_Pro753del	c.2238_2258del	
		p.Glu746_Ser752del	c.2236_2256del	
	Del 24	p.Ser752_Ile759del	c.2253_2276del	
20	T790M	p.Thr790Met	c.2369C>T	T790M
	S768I	p.Ser768Ile	c.2303G>T	S768I
	InsG	p.Asp770_Asn771insGly	c.2310_2311insGGT	Exon 20 insertion
	InsASV	p.Val769_Asp770insAlaSerVal	c.2307_2308insGCCAGCGTG	
			c.2309_2310delinsCCAGCGTGGAT	
	InsSVD	p.Asp770_Asn771insSerValAsp	c.2311_2312insGCGTGGACA	
	InsH	p.His773_Val774insHis	c.2319_2320insCAC	
21	L858R	p.Leu858Arg	c.2573T>G	L858R
			c.2573_2574delinsGT	
			c.2573_2574delinsGA	
	L861Q	p.Leu861Gln	c.2582T>A	L861Q



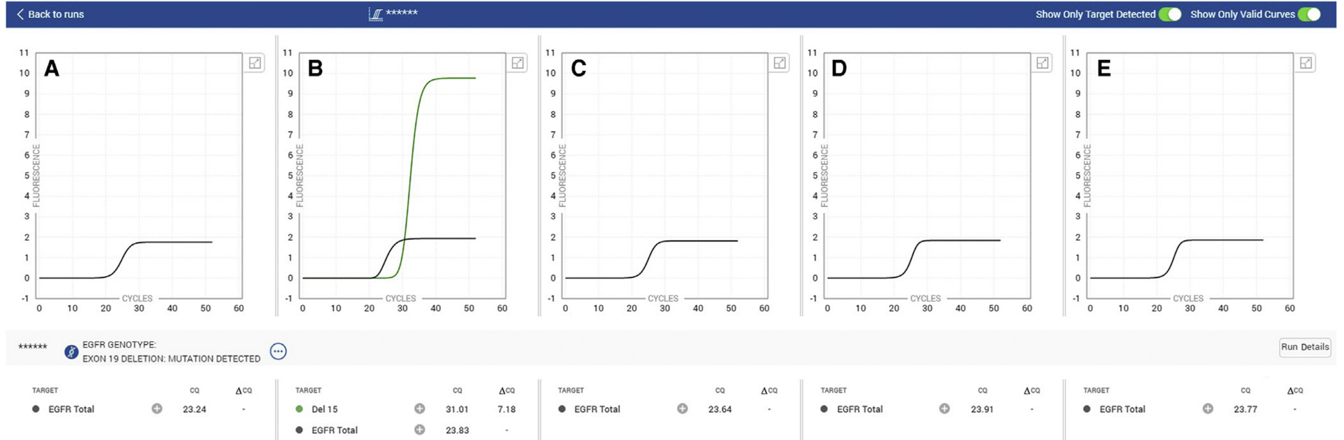


Figure 1 Idylla Explore version 2.5.1294.1 (Biocartis, Mechelen, Belgium) default display of a sample with an exon 19 mutation (deletion 15) present: detail of PCR curves and cycle of quantification (Cq) values. Idylla Explore display of a sample with an exon 19 mutation present. **A:** Details of PCR curves and Cq values; Cq values are available for the sample processing controls, EGFR Total, and the target for which a signal has been detected (default view); black PCR curves represent the EGFR Total, and green PCR curve represents the EGFR exon 19 target [deletion 15 (Del 15)]. **B:** Detail of PCR curves (Idylla Explore default view). The letters A to E in the image reflect the five PCR chambers in the Idylla cartridge.

sections (mostly one, up to six) had a thickness of 5 or 10 μm and were sampled within the same FFPE block and mostly consecutive to the sections used before to generate the reference result with the routine method. Tumor content and area of the specimen were determined on hematoxylin-eosin–stained slides by a pathologist and, if needed, macrodissection was performed to achieve a neoplastic cell content of at least 10%. The FFPE tissue sections were placed directly into the Idylla cartridge following the assay instructions of the manufacturer (Biocartis).

Artificial FFPE samples (ie, FFPE Reference Standards from Horizon Discovery, Cambridge, UK) were included as external controls: all 15 sites tested *EGFR* L858R and T790M, both at a 20% allelic frequency.

Idylla EGFR Mutation Assay Description

The Biocartis Idylla EGFR Mutation Assay, performed on the Idylla System, is an assay intended for the qualitative detection of 51 different *EGFR* oncogene mutations: exon 18 (G719A/C/S), exon 19 (deletions), exon 20 mutations (T790M, S768I, and insertions), and exon 21 (L858R and L861Q). The detected *EGFR* mutations and the corresponding Idylla EGFR Mutation Assay genetic calls are presented in Table 1. In this study, the Idylla EGFR Investigational Use Only Assay has been used, as the CE-IVD–labeled Idylla EGFR Mutation Test was not yet on the market. The Idylla EGFR Mutation Assay has, in contrast to the Idylla EGFR Mutation Test (IVD), no performance claims.

The Idylla EGFR Mutation Assay uses FFPE tissue sections originating from NSCLC lesions, with a minimum input of one FFPE section (5 μm thick) containing at least 10% of neoplastic cells. The time of completion from FFPE tissue sample to test result (not including macrodissection, if any) is approximately 2.5 hours, with <2 minutes hands-on time. In the cartridge, the entire process from FFPE sample to result, including fully integrated sample preparation, liberation of nucleic acids, and real-time PCR amplification and detection, is covered. Briefly, after insertion of the FFPE tissue section into the cartridge, deparaffinization, disruption of the tissue, and lysis of the cells are induced by a combination of chemical reagents, enzymes, heat, and high-intensity focused ultrasound. The resulting liberated

Table 2 Comparison between Results of the Idylla EGFR Mutation Assay and Results of Routine Reference Methods (15 Sites, 449 NSCLC Samples)

		Reference methods									
		G719A/C/S	Ex19del	T790M	S768I	Ex20ins	L858R	L861Q	G719A/C/S + S768I	Ex19del + T790M	Ex19del + L858R
Idylla	G719A/C/S	5									
	Ex19del		87							1	
	T790M										
	S768I				2				1		
	Ex20ins					6					
	L858R						52				1
	L861Q							7			
	G719A/C/S + S768I								9		
	Ex19del + T790M		1							18	
	Ex19del + L861Q										
	L858R + T790M										
	L858R + S768I						1				
	L861Q + T790M							1			
	G719A/C/S + S768I + L861Q										
	No mut detected		6				2	1			
	Invalid/error		3[‡]								
	Total	5	97	0	2	6	55	9	10	19	1

(*table continues*)

Routine reference methods: Ion Torrent AmpliSeq Colon and Lung Cancer Research Panel version 2 (Thermo Fisher Scientific), Oncomine panel (Thermo Fisher Scientific), HaloPlex Cancer Research Panel (Agilent Technologies), in-house—developed next-generation sequencing, therascreen EGFR Pyro Kit (Qiagen), in-house—developed pyrosequencing, cobas EGFR Mutation Test version 2 (Roche), therascreen EGFR RGQ PCR Kit version 1 or 2 (Qiagen), MassArray Lung Cancer Panel (Diatech), and Sanger sequencing. Values in parentheses were not included in the data set used for concordance analysis.

[*]*n* = 16 (Discordant by design).

[†]Exon 19 (7), exon 20 (4), and exon 21 (1).

[‡]*n* = 6 (invalid/error).

Ex19del, exon 19 deletion; Ex20ins, exon 20 insertion; mut, mutation; NSCLC, non—small-cell lung cancer.

nucleic acids are then analyzed in five parallel multiplex PCRs by means of highly selective target amplification primers and using fluorescently labeled probes to detect target sequences. To this end, the cartridge contains allele-specific primers and probes and all necessary PCR reagents present in a stable formulation. A conserved fragment in the transmembrane region of the *EGFR* gene is amplified simultaneously and serves as a sample processing control (named EGFR Total) to check for adequate execution of the complete process and as a measure for the amount of amplifiable DNA in the sample.

The resulting fluorescence signals are analyzed in the Idylla System by EGFR-specific software and translated into genetic calls. To this end, the fluorescence signals are evaluated for PCR curve validity; and for each valid curve, a cycle of quantification value (Cq) is calculated. The presence of a mutant genotype is determined by calculating the difference between the EGFR Total Cq and the Cq obtained for the mutant signal(s) (ie, the ΔCq). A ΔCq value within a predefined validated range defines a valid mutant signal (mutation detected). Samples with a valid EGFR Total signal but a ΔCq value outside the predefined range for a mutant signal are reported as mutation negative (no mutation detected). In case no EGFR Total signal was detected, no mutant result can be determined and the result for that PCR is considered invalid. Invalid calls might be due to insufficient DNA input, severe DNA fragmentation (potentially caused by extended fixation time), the presence of inhibitors in the sample, incorrect placement of a sample in the cartridge, incorrect storage of the cartridge, use of a cartridge that exceeded its in-use period after removal from the pouch, or cartridge malfunctioning. For the genotype calls that cover several mutations (eg, G719A/C/S, exon 19 deletion, and exon 20 insertion), the genotype call will be reported as positive (mutation detected) if at least one valid mutation signal is detected, regardless of the status (valid/invalid) of the other mutations within the genotype call. For the Idylla EGFR Mutation Assay to be invalid, all seven genotypes must contain an invalid result. A result reporting the presence, absence, or invalidity for each mutation or mutation group in the *EGFR* gene in the analyzed sample is displayed on the Idylla Console screen, as well as an average of the Cq values of the EGFR Totals (present in each of the five multiplex PCRs). If the Idylla report shows a mix of invalid and valid genotype calls, the robustness for the valid genotype call can be ensured. Each PCR chamber

Table 2 (*continued*)

Reference methods

L858R + T790M	L858R + S768I	L858R + G719A/C/S	G719A/C/S + S768I + L861Q	Ex19del + T790M + other mut	Ex19del + L861Q + T790M	L858R + other mut	T790M + other mut	Other mut	No mut detected	Invalid/error	Total
									1		6
											88
							1*				1
									2		5
											6
3	1	1				2*					60
									1		8
											9
				1*							20
					1						1
6											6
	3										4
											1
			1								1
								12*†	206	1‡	228
									1‡	1‡	5
9	4	1	1	1	1	2	1	12	211	2	449

in the EGFR cartridge is individually validated for performance by means of the sample processing control. If a sample processing control signal of a PCR chamber is valid, all results of the PCR chamber are considered valid, independent of the performance of the other chambers.

The Idylla Explore application offers visualization of PCR curves and the corresponding Cq values from the Idylla EGFR Mutation Assay results (Figure 1). This is a visualization tool and not intended to be used for data interpretation.

Reference Methods Used for Routine Analysis of EGFR Mutations

The following reference methods were used for routine analysis of the *EGFR* mutational status: NGS using the Ion Torrent AmpliSeq Colon and Lung Cancer Research Panel version 2 (Thermo Fisher Scientific, Waltham, MA; $n = 12$ samples), the Oncomine Focus Assay (Thermo Fisher Scientific; $n = 25$), the HaloPlex Cancer Research Panel (Agilent Technologies, Santa Clara, CA; $n = 20$), or in-house−developed NGS ($n = 3$); pyrosequencing using the therascreen EGFR Pyro kit (Qiagen, Venlo, the Netherlands; $n = 6$) or in-house−developed pyrosequencing ($n = 34$); PCR-based assays using the cobas EGFR Mutation Test version 2 (Roche Molecular Systems, Pleasanton, CA; $n = 130$) or the therascreen EGFR RGQ PCR Kit version 1 or 2 (Qiagen; $n = 71$); mass spectrometric methods using the MassArray Lung Cancer Panel (Diatech, Jesi, Italy; $n = 30$); and Sanger sequencing using in-house methods ($n = 118$). Commercial assays were performed according to the manufacturer's instructions. Analytical sensitivities for the commercial assays can be found in the respective manufacturer's instructions. In-house methods were performed by adopting different protocols and equipment at each site.

Methods Used in Further Analysis of Discordant Samples

Digital droplet PCR (ddPCR) of DNA retrieved from the Idylla EGFR Mutation Assay cartridge, of DNA used for the reference method, and/or of FFPE tissue sections was performed on a BioRad (Hercules, CA) QX100 system, according to the manufacturer's instructions.

Other methods used in further analysis were performed as described above.

Statistical Analysis

Agreement between Idylla and the comparator method was evaluated on the basis of point estimates for overall, positive, and negative percentage diagnostic agreement together with 95% one-sided Wilson score CIs.

Table 3 Other Mutations of *EGFR* Detected by Routine Reference Methods (Discordant by Design)

Samples	Idylla results	Routine reference method results	Routine reference method used
11_03	ND	Exon 19; c.2270A>G; p.L757R	therascreen EGFR Pyro Kit
11_05	Exon 19; deletion	Exon 19; deletion c.2235—2249; p.E746-A750del	Sanger sequencing
	Exon 20; T790M	Exon 20, c.2369 C>T; p.T790M	
	ND	Exon 20; c.2390G>C; p.C797S	
11_07	ND	Exon 20; c.2311_2319dupAACCCCTAC p.N771_H773dup; c.2317C>T p.H773Y	Sanger sequencing
11_09	ND	Exon 20; c.2335_2336delinsTT; p.G779F	Sanger sequencing
07_04	ND	Exon 19; deletion c.2236_2253delinsGTTAAT	Sanger sequencing
14_05	ND	Exon 19; c.2250_2276delinsCTC; p.T751_I759delinsS	Sanger sequencing
14_18	ND	Exon 19, c.2240_2264delinsCGAGAGA; p.L747_A755delinsSRD	Sanger sequencing
14_25	ND	Exon 19del; c.2252_2276delinsN; p.T751_I759delinsN	Sanger sequencing
14_28	Exon 21; L858R	Exon 21; c.2573T>G; p.L858R	Sanger sequencing
	ND	Exon 18; c.2126A>C; p.E709A	
10_15	ND	Exon 20; c.2309_2317dup; p.D770_H773dup	In-house NGS
13_28	ND	Exon 20; c.2294_2295insGATGGC; p.V765_M766insMA	cobas EGFR Mutation Test version 2
12_03	Exon 21; L858R	Exon 21; c.2573T>G; p.L858R	MassArray Lung Cancer Panel
	ND	Exon 18; p.E709G	
12_23	ND	Exon 21; p.L833V	MassArray Lung Cancer Panel
	ND	Exon 21; p.H835L	
03_05	ND	Exon 19; c.2252_2276del25insA; p.T751_I759delinsN	therascreen EGFR RGQ PCR Kit
03_10	Exon 21; T790M	Exon 21; c.2369C>T; p.T790M	therascreen EGFR RGQ PCR Kit
	ND	Exon 19; c.2252_2276del25insA; p.T751_I759delinsN	
03_16	ND	Exon 19; c.2246_2260del15; p.A750_K754del	therascreen EGFR RGQ PCR Kit

ND, not detected.

Results

Verification with External Control Samples

At all 15 sites, the *EGFR* L858R and T790M (both at a 20% allelic frequency) artificial FFPE samples (ie, FFPE Reference Standards from Horizon Discovery) were included as external controls. The Idylla EGFR Mutation Assay, independently performed at 15 sites, identified each of these two mutations correctly; and, therefore, it can be concluded that interlaboratory reproducibility of the external control samples was 100%.

Performance of Idylla EGFR Mutation Assay on Clinical Samples

Using the Idylla EGFR Mutation Assay, the *EGFR* mutational status of archived clinical FFPE tissue sections, originating from NSCLC lesions from 449 patients, was determined at 15 centers (Supplemental Table S1). The Idylla results were compared with the original results made by routine reference methods (*Materials and Methods*). Macrodissection was performed in 117 of the 449 cases to increase the percentage of tumor area, as recommended in the assay instructions for samples with low tumor cellularity.

The Idylla EGFR Mutation Assay identified EGFR mutations in 220 samples (Table 2). Deletions in exon 19 were detected in 109 samples, 21 of which were associated with one other mutation detected by the Idylla EGFR Mutation Assay. The L858R mutation was reported in 70 samples, and in 10 of these samples L858R was associated with one other mutation. A G719A/C/S mutation was detected in 16 samples, of which 10 were associated with one or two other mutations. Eleven samples harbored an L861Q mutation, and three of them were associated with one or two other mutations. Among the 19 samples bearing an S768I mutation, 14 were associated with one or two other mutations. T790M was detected in 28 samples, of which 27 were associated with one other EGFR variant. Finally, exon 20 insertions were found in six samples.

In 16 samples, routine reference methods identified rare mutations of EGFR that were not detected by the Idylla EGFR Mutation Assay (Tables 2 and 3). The negative Idylla results could, however, be expected as the involved mutations are not within the scope for which the Idylla EGFR Mutation Assay has been designed. Therefore, these 16 samples were excluded from the analysis as discordant by design.

An invalid or error call was reported in four cases with the Idylla EGFR Mutation Assay (samples 02_06, 10_11, 08_13, and 08_17), in one sample with a routine reference method (sample 01_25, failing both the Ion Torrent AmpliSeq Colon and Lung Cancer Research Panel version 2 and the cobas EGFR Mutation Test version 2), and in one sample with an invalid call for both the Idylla EGFR Mutation Assay and the therascreen EGFR RGQ PCR Kit, used as a routine reference method

(sample 08_19). These six samples were excluded from the data set.

As a result, the data set contained 427 (449 − 16 − 6) samples for concordance analysis. Overall, in this first assessment, without including the results of a third method analysis, there was complete agreement between the Idylla results and results of the routine reference methods in 402 cases of the 427 samples (overall concordance of 94.15%; 95% CI, 91.50%−96.00%).

Of the 427 cases, 25 discordant test results were observed (Table 2). Of these 25 discordant samples, 13 were considered as wild-type/mutant discordants (Table 4). For the other 12 samples, there was a difference in number of detected mutations; these are so called mutant/mutant + 1 discordants (Table 5).

Overall, for 8 of the 25 discordant samples, the Idylla EGFR Mutation Assay detected a mutation that was not found by the routine reference method (negative percentage agreement, 96.26%); and in 17 cases, the Idylla EGFR Mutation Assay did not detect the reported mutation by the routine reference method (positive percentage agreement, 92.02%).

Further Evaluation of the Discordant Results

Further analysis of the 25 discordant samples was performed by analyzing new FFPE section material or, to circumvent the low availability of FFPE material from NSCLC lesions, by analyzing the DNA retrieved from the Idylla cartridge or DNA as used for the reference method. However, for eight samples (14_16, 01_11, 07_06, 14_11, 14_24, 01_18, 03_17, and 03_04) (Tables 4 and 5), the DNA input was too low for additional analysis. As a consequence, the test result for these samples was inconclusive; and the eight samples were therefore excluded from the data set for the final concordance determination.

For samples 07_16 and 07_17 (Table 4), the exon 19 deletion was not detected on a new FFPE section with a ddPCR technique. For sample 07_16, DNA was also retrieved from the Idylla cartridge and analyzed by ddPCR; and this test also did not result in the detection of the exon 19 deletion. However, the exon 19 deletion was detected by another Idylla EGFR Mutation Assay on the DNA material previously extracted and used for the reference method. Hence, both samples were classified as inconclusive and removed from the data set because it was suspected that the inability to confirm the exon 19 deletion identified by the routine reference method only was due to sample heterogeneity.

As to sample 05_32, the therascreen EGFR RGQ PCR Kit only identified mutation S768I, whereas the Idylla EGFR Mutation Assay identified mutation G719A/C/S in addition. As both results were clinically concordant, further analysis was not performed on this sample. Therefore, this sample was classified as inconclusive and removed from the data set.

In summary, 11 of the 25 discordant samples were removed from the data set. The next paragraphs describe in detail the further analysis of the discordant samples.

Wild-Type/Mutant Discordant Samples

In 13 of 25 discordant samples, the Idylla EGFR Mutation Assay identified mutations that were not detected by the routine reference methods, or the other way around (Table 4). Of these 13 samples, 9 were inconclusive (samples 14_16, 01_11, 07_06, 07_16, 07_17, 14_11, 14_24, 01_18, and 03_17), as explained in the previous paragraph.

Among the four remaining wild-type/mutant discordant samples (01_08, 04_23, 14_12, and 10_08), the Idylla EGFR Mutation Assay identified mutation L861Q in sample 04_23, whereas the cobas EGFR Mutation Test version 2 did not. The presence of L861Q was confirmed by ddPCR on FFPE material as well as on DNA isolated from the cartridge. The presence of mutation S768I in sample 01_08, as detected by the Idylla EGFR Mutation Assay, was not confirmed by ddPCR and NGS analysis of cartridge and of FFPE material. The L861Q mutation, found earlier by Sanger sequencing in sample 14_12, was confirmed by ddPCR at an allelic frequency of 4%, which is below the Idylla EGFR Mutation Assay limit of detection. The DNA isolated from the cartridge of sample 10_08 was tested by NGS; and the result confirmed the exon 19 deletion c.2240_2257delTAAGAGAAGCAACATCTC, detected before by pyrosequencing, at 57.74% allelic frequency. The Idylla EGFR Mutation Assay did not identify this deletion in this sample. The report of the NGS data revealed that a second low-prevalence mutation was present in the sample (ie, c.2264C>G). This mutation interferes with the primer/probe annealing of the Idylla exon 19 deletion assay, resulting in a substantial decreased sensitivity of the detection chemistry and the failure to detect the exon 19 deletion with the Idylla EGFR Mutation Assay.

Mutant/Mutant + 1 Discordant Samples

In 12 samples, an additional mutation was detected in either the Idylla EGFR Mutation Assay or the routine reference method; these samples are designated mutant/mutant + 1 discordant samples (Table 5). Confirmation with ddPCR of the three mutations detected with routine reference methods in sample 03_04 was inconclusive, due to less than minimal DNA input. Sample 05_32 was considered inconclusive as no further analysis was performed. Both samples were, therefore, excluded from the data set.

As to the remaining 10 mutant/mutant + 1 discordant samples, further analyses confirmed the results found by the Idylla EGFR Mutation Assay in three samples (samples 09_12, 08_26, and 06_16). The Idylla EGFR Mutation Assay identified mutation T790M in samples 09_12 and 08_26, whereas the cobas EGFR Mutation Test version 2 and the Qiagen therascreen EGFR RGQ PCR Kit did not,

Table 4 Wild-Type/Mutant Discordant Results between the Idylla EGFR Mutation Assay and Routine Reference Methods

Sample	Baseline/progression	FFPE tissue section, μm	FFPE tissue sections put in Idylla cartridge, *n*	Tumor cells, %	Tumor area, mm^2
14_16	Progression	5	1	80	10
01_08	Baseline	5	1	10	0.04
01_11	Baseline	5	1	30	100
04_23	Baseline	5	2	50	18
07_06	Baseline	10	1	60	90
07_16	Baseline	10	1	80	30
07_17	Baseline	10	1	75	27
14_11	Baseline	10	1	10	5
14_12	Baseline	5	1	50	15
14_24	Baseline	5	1	>50	40
10_08	Baseline	5	2	>50	4
01_18	Baseline	5	1	85	100
03_17	Baseline	10	1	40	1

(*table continues*)

*Sanger sequencing.
†Digital droplet PCR.
‡cobas EGFR Mutation Test version 2 (Roche).
§Ion Torrent AmpliSeq Colon and Lung Cancer Research Panel version 2 (Thermo Fisher Scientific).
¶NGS Oncomine panel (Thermo Fisher Scientific).
‖In-house pyrosequencing.
**In-house next-generation sequencing.
††therascreen EGFR RGQ PCR Kit (Qiagen).
—, To be excluded from data set; C, concordant; D, discordant; Ex19del, exon 19 deletion; FFPE, formalin fixed, paraffin embedded; LOD, limit of detection; WT, wild type.

respectively. ddPCR analysis on DNA from the cartridge confirmed the presence of T790M at a low allelic frequency (2.4% in sample 09_12 and 0.5% in sample 08_26). The exon 19 deletion, reported by the cobas EGFR Mutation Test version 2 in sample 06_16, was not confirmed by ddPCR. Therefore, the Idylla EGFR Mutation Assay result for the latter sample was classified as concordant.

In seven remaining samples, the results of the third method used (ddPCR analysis) were not concordant with the results obtained with the Idylla EGFR Mutation Assay; and these samples were, thus, considered discordant. The S768I mutation in sample 04_20, which was only detected by the Idylla EGFR Mutation Assay and not by the cobas EGFR Mutation Test version 2, was not confirmed by ddPCR on DNA material from the cartridge or by ddPCR analysis of FFPE material, but the latter was hindered by low DNA content. In sample 13_08, the Idylla EGFR Mutation Assay could not identify the mutation G719A/C/S, detected by the cobas EGFR Mutation Test version 2. Using ddPCR, G719S, at an allelic frequency of 0.15%, was detected on DNA retrieved from the Idylla cartridge, whereas in FFPE material, the same mutation was found at 0.25%. The T790M mutation in sample 07_21, determined before by Sanger sequencing only, was confirmed by ddPCR analysis on a new FFPE tissue section. The allelic frequency was 0.5%. As Sanger sequencing needs at least 10% to 15% mutated DNA to be detected, sample heterogeneity might have contributed to this discordance. In samples 13_27 and 13_30, the T790M mutations, identified by the cobas EGFR Mutation Test version 2 but not by the Idylla EGFR Mutation Assay, were confirmed by ddPCR analysis, showing the presence of T790M in both samples at allelic frequencies (approximately 1% to 2%). The presence of mutation T790M, found in sample 12_09 when using the MassArray Lung Cancer Panel and not with the Idylla EGFR Mutation Assay, was confirmed by ddPCR at 2.59% allelic frequency in FFPE material and in DNA from the cartridge. A ddPCR analysis of sample 03_09 on DNA derived from the Idylla cartridge was inconclusive because of insufficient material, although ddPCR on FFPE material found an S768I mutation at 30% allelic frequency. Unlike the earlier Sanger sequencing and therascreen EGFR RGQ PCR Kit results, the original Idylla EGFR Mutation Assay did not find this mutation. Considering the high allelic frequency of the mutation, a retest of the Idylla EGFR Mutation Assay on FFPE material was performed; and the S768I was correctly reported, which could indicate sample heterogeneity.

Performance of Idylla EGFR Mutation Assay

Using the results of the further analysis, as outlined above, 11 samples were removed from the data set of 427 samples (samples 14_16, 01_11, 07_06, 07_16, 07_17, 14_11, 14_24, 01_18, 03_17, 05_32, and 03_04), bringing the total number of samples down to 416. As well, of the remaining

Table 4 (*continued*)

Macrodissection	Idylla	Routine reference method	Further analysis	Conclusion based on further analysis
No	G719A/C/S	No mutation detected*	Inconclusive†	—
No	S768I	No mutation detected‡§	WT†§	D
No	S768I	No mutation detected§	Inconclusive†§	—
No	L861Q	No mutation detected‡	L861Q†	C
Yes	No mutation detected	Ex19del*	Inconclusive†	—
Yes	No mutation detected	Ex19del¶	Inconclusive†	—
Yes	No mutation detected	Ex19del¶	Inconclusive†	—
No	No mutation detected	L858R*	Inconclusive†	—
No	No mutation detected	L861Q*	4% L861Q†	D, L861Q<LOD
No	No mutation detected	L858R*	Inconclusive†	—
Yes	No mutation detected	Ex19del‡‖	Ex19del†**	D
No	No mutation detected	Ex19del‡	Inconclusive†§	—
No	No mutation detected	Ex19del*††	Inconclusive†	—

14 discordant samples, four (samples 06_16, 09_12, 04_23, and 08_26) were reclassified as concordant, reducing the total number of discordant samples to 10 (Tables 4 and 5). Taking into account the decreased number of samples in the data set and the increased number of concordant samples, the findings of the Idylla EGFR Mutation Assay were in agreement with the confirmed reference method results in 406 of 416 samples. Consequently, the overall concordance with routine reference methods, including further analysis, was found to be 97.59% (95% CI, 95.63%−98.69%), with a negative percentage agreement of 96.26% (95% CI, 92.80%−98.09%) and a positive percentage agreement of 99.01% (95% CI, 96.46%−99.73%).

Influence of Tissue Size on Idylla EGFR Mutation Assay Result

The amount of tissue needed to enable a valid Idylla EGFR Mutation Assay is small. For example, a tissue surface area of 0.43 mm^2 (sample 09_09) still gave a valid result that was concordant with the earlier result of the routine reference method. There was neither a relationship between the total tissue surface area and the validity of the Idylla EGFR Mutation Assay nor between the total tissue surface area and the concordance of the Idylla result with the routine reference method result (Table 6). This hypothesis was statistically confirmed using a χ^2 statistic: P value for validity of 0.63 and P value for concordance of 0.11.

Discussion

The detection of *EGFR* mutations in NSCLC, either activating mutations in newly diagnosed NSCLC patient or resistance mutations after first-generation EGFR TKI treatment, is relevant because the latest guidelines by the National Comprehensive Cancer Network and the European Society for Medical Oncology recommend EGFR TKIs as first-line therapy for patients with advanced NSCLC harboring activating EGFR mutations.[18−21]

Numerous methods are available for the detection of EGFR mutations, which necessitate many steps (deparaffinization, DNA extraction, PCR amplification, and result interpretation) in a specific infrastructure with expert staff to perform analyses and interpret the results; they usually take more than a day from sample to result. Moreover, the current routine methods used for the detection of EGFR mutations are often performed batch wise, with several samples simultaneously, to be as cost effective as possible. This will inevitably delay turnaround time from sample reception to final result. The Idylla EGFR Mutation Assay, however, requires minimal infrastructural measures and is designed to run on a patient-per-patient base, which reduces turnaround time dramatically. It could, therefore, be useful to answer urgent requests.[29,30]

The current study compared the Idylla EGFR Mutation Assay results with the original results obtained by routine reference methods for the detection of EGFR mutations in FFPE tissue samples. Each of the 15 participating clinical centers included at least one NSCLC FFPE sample for each of the seven Idylla EGFR Mutation Assay genotype calls indicated in Table 1. Therein, the mutation rates obtained in this study are not representative for the incidence of EGFR mutations in the general NSCLC patient population.

In total, 449 NSCLC FFPE samples were selected and tested. Samples that were discordant by design (n = 16) or obtained an invalid test result (n = 6) with either the routine method or the Idylla EGFR Mutation Assay were excluded.

Table 5 Mutant/Mutant + 1 Discordant Results between the Idylla EGFR Mutation Assay and Routine Reference Methods

Sample	Baseline/progression	FFPE tissue section, μm	FFPE tissue sections put in Idylla cartridge, *n*	Tumor cells, %	Tumor area, mm^2
09_12	Progression	5	1	30	3.19
04_20	Baseline	5	1	20	12
08_26	Baseline	5	5	>10	25
05_32	NA	5	1–4	60	∼15
06_16	Baseline	10	6	15	4
07_21	Baseline	10	1	50	50
13_08	Baseline	5	1	50	100
13_27	Progression	5	2	20	200
13_30	Progression	5	2	30	15
12_09	NA	5	1	>50	100
03_04	Progression	10	1	75	2
03_09	Baseline	10	1	50	2

(*table continues*)

*cobas EGFR Mutation Test version 2 (Roche).
†Digital droplet PCR.
‡therascreen EGFR RGQ PCR Kit (Qiagen).
§Idylla EGFR Mutation Assay retest.
¶MassArray Lung Cancer Panel (Diatech).
‖Sanger sequencing.
—, To be excluded from data set; C, concordant; D, discordant; Ex19del, exon 19 deletion; FFPE, formalin fixed, paraffin embedded; Inconcl, inconclusive; LOD, limit of detection; NA, information not available; ND, not determined.

The fact that the mutants identified by the reference method but classified as discordant by design have not been evaluated is a limitation of the Idylla EGFR Mutation Assay because these mutants are rarely observed in large cohorts of NSCLC and not of clinical importance.[31–33]

As a result, the final data set contained 427 samples for concordant analysis. Discordance between results of the Idylla EGFR Mutation Assay and routine reference methods was observed for 25 of these 427 samples. The overall concordance between the Idylla EGFR Mutation Assay and the routine reference methods, before third method analysis, was 94.14% (95% CI, 91.50%–96.00%). Further evaluation of the discordant results obtained revealed a final overall concordance of 97.59% (95% CI, 95.63%–98.69%). The 25 discordant results were investigated in depth and described fully in the *Results* section. Some of the discordant results can be explained by the low allelic frequencies of the present mutations, which were below the limit of detection of the assays. The discordances were divided into 13 wild-type/mutant discordant samples (ie, the Idylla EGFR Mutation Assay identified mutations not detected by the routine reference methods or the other way around) and 12 mutant/mutant + 1 discordant samples (ie, the Idylla EGFR Mutation Assay detected an additional mutation compared with the routine reference method or the other way around).

The further discordant analyses were troubled (11 samples needed to be excluded) by two well-known root causes: the low availability of FFPE material from NSCLC lesions and tumor/sample heterogeneity. It is generally accepted that tissue samples obtained from NSCLC patients are mostly small biopsies obtained by endoscopy or by image-guided transthoracic core-needle biopsy.[34] After histologic analysis, which consumes numerous sections, and molecular biology analyses performed with the routine reference method used in the laboratory, usually only a small amount of tissue sample material remains. Also, in this study, in eight discordant cases, the FFPE materials remaining were not sufficient to obtain results from further analysis with additional methods and, thus, unfortunately these samples needed to be excluded from the final concordance data set as inconclusive. Second, tumor sample heterogeneity is an issue in comparative studies.[35,36] Sample heterogeneity was a possible issue in two cases where the Idylla EGFR Mutation Assay did not detect the exon 19 deletion detected with the routine reference method; using a sensitive technique (ddPCR), detection of this mutation in DNA retrieved from the cartridge (performed for one of the two samples) and on a new FFPE section was also unsuccessful; however, the Idylla EGFR Mutation Assay was able to detect the mutation on the DNA material previously extracted and used for the reference method. These two inconclusive samples were, therefore, also removed from the final concordance data set. Finally, one sample was considered inconclusive as no further analysis was performed.

The final overall concordance between the Idylla EGFR Mutation Assay and the confirmed reference routine test results was, therefore, found to be 97.59% (95% CI, 95.63%–98.69%), with a negative percentage agreement of 96.26% (95% CI, 92.80%–98.09%) and a positive percentage agreement of 99.01% (95% CI, 96.46%–99.73%),

Table 5 (*continued*)

Macrodissection	Idylla	Routine reference method	Further analysis	Conclusion based on further analysis
No	Ex19del + T790M	Ex19del*	Ex19del + 2.4% T790M†	C, T790M<LOD
No	L858R + S768I	L858R*	L858R†	D
No	L861Q + T790M	L861Q‡	L861Q + 0.5% T790M†	C, T790M<LOD
Yes	S768I + G719A/C/S	S768I§	ND	—
No	L858R	L858R + Ex19del*	L858R†	C
Yes	L858R	L858R + T790M§	L858R + 0.5% T790M†	D, T790M<LOD
No	L858R	L858R + G719A/C/S*	L858R + 0.15% G719 Sv	D, G719S<LOD
No	L858R	L858R + T790M*	L858R + 1–2% T790M†	D, T790M<LOD
No	L858R	L858R + T790M*	L858R + 1.26% T790M†	D, T790M<LOD
No	Ex19del	Ex19del + T790M¶	ex19del + 2,59% T790M†	D, T790M<LOD
Yes	ex19del + L861Q	ex19del + L861Q + T790M‡‖	Inconcl†	—
No	L858R	L858R + S768I‡‖	L858R + S768I§	D

demonstrating the good performance of the rapid Idylla EGFR Mutation Assay.

Four discordant samples were reclassified as concordant because third method analyses with either ddPCR or NGS confirmed the mutations identified with the Idylla EGFR Mutation Assay but not with the routine reference method. Six other discordant samples exhibited L861Q, G719S, or T790M mutations, according to the reference results. Third method analysis revealed that the observed mutant allelic frequencies were low (<5%), which can explain why the Idylla EGFR Mutation Assay was not able to detect the mutants at the given sample input; also, sample heterogeneity might have played a role.

For baseline testing at diagnosis of metastatic NSCLC, the presence or absence of additional sensitizing mutations does not influence therapy guidance. Of the 10 analytically discordant samples, four baseline samples could, therefore, be considered clinically concordant. On the contrary, for samples tested at progression, the presence or absence of an additional sensitizing mutation or of the T790M resistance mutation can change therapy guidance.[18] Two of the analytically discordant samples taken at progression had a discordant result at the level of the T790M mutation and should, therefore, be considered clinically discordant. The remaining four analytically discordant samples were also considered clinically discordant because for two of them (samples 14_12 and 12_09) the baseline/progression status was unknown, and the two other samples (samples 01_08 and 10_08) were wild-type/mutant discordant samples. Concerning the T790M mutant, it can be concluded that the Idylla EGFR Mutation Assay missed the detection of low mutant allelic frequency T790M (ie, <5%). Deamination in FFPE samples is known to cause baseline noise in NGS data.[37] T790M is a target that is affected by deamination and, therefore, entails the risk of false-positive calls due to deamination issues. In the Idylla EGFR Mutation Assay, the sensitivity for T790M is, therefore, by design limited to detect a mutant allelic frequency of ≥5% to reduce this risk.

As discussed in the previous paragraph, several factors contribute to the fact that frequently only a small amount of tissue sample remains to perform a molecular analysis to get a conclusive result. Fortunately, the Idylla EGFR Mutation Assay only needs a small amount of FFPE tissue sample. Even with <10 mm^2 of tissue area, the Idylla EGFR Mutation Assay gave a valid result in 99.0% of the cases. Recently, also, De Luca et al[38] showed that 80% of the samples that failed on NGS could be successfully tested with the Idylla EGFR Mutation Test. Furthermore, in the current study, it was shown that the percentage of valid results was not related to the sample size. In the same manner, there was no relationship between the size of the tissue area and the concordance of the Idylla EGFR Mutation Assay result with the routine reference method result, which was comprised between 91.9% and 100.0%.

Taken together, the results of this comparative study confirm the high sensitivity and specificity of the Idylla EGFR Mutation Assay and its ability to provide valid and concordant results starting from small tissue samples. However, as counterargument, it can be challenged that for samples of which not sufficient material could be recovered for third method analysis, and which were in this study classified as inconclusive, the Idylla EGFR Mutation Assay was not able to detect a mutation, whereas the routine method did. As also mentioned in the instructions for use of the Idylla EGFR Mutation Assay, when a no mutation detected result for all seven EGFR genotypes has been obtained, the presence of a mutation in the seven EGFR genotypes might not be excluded because the result is dependent on the integrity of the specimen DNA, the percentage of mutant alleles present in the specimen, the

Table 6 Influence of Tissue Size on Validity of the Idylla EGFR Mutation Assay and Concordance with the Reference Method Result

Total tissue area, mm^2	Valid, *n*/total (%)	Concordant, excluding invalids, *n*/total (%)
<10	97/98 (98.98)	90/97 (92.78)
≥10 and <25	74/74 (100.00)	68/74 (91.89)
≥25 and <300	234/238 (98.32)	227/234 (97.01)
≥300	23/23 (100.00)	23/23 (100.00)

For five samples, the sample size was not recorded; samples that were discordant by design ($n = 16$) are not included in this table. The exact number of sections put in the cartridge was only recorded for 403 samples.

absence of inhibiting substances, and the presence of sufficiently amplifiable DNA. The average Cq of the EGFR control, depicted on the report, gives an indication of the quantity of amplifiable DNA present in the sample and is linked to the analytical sensitivity. Thus, it can be concluded that when insufficient amplifiable DNA is present in the cartridge, it will affect the analytical sensitivity of the test and logically the risk for a false-negative result might be higher.

Although the Idylla EGFR Mutation Assay is not intended to detect all possible EGFR mutations or to quantify allelic frequency, this multicenter performance study demonstrates that the test can currently detect all the most prevalent and clinically relevant EGFR mutations. A limitation of the mutation coverage can be that the EGFR C797S mutation is not included in the current Idylla EGFR Mutation Assay. This mutation is reported to be a mechanism of resistance to the third-generation inhibitors targeting T790M. Fourth-generation EGFR TKIs, targeting the C797S mutation, are not yet available. Nevertheless, other molecular techniques could be used to either quantify the EGFR mutation detected, if necessary, or to detect other mutations of EGFR or to simultaneously detect the mutation status of other potentially relevant tumor biomarkers.

Finally, although not included as outcome in this study design, an important advantage of the rapid Idylla EGFR Mutation Assay is the rapid turnaround time. Because the detection of the *EGFR* mutational status could be pressing for patients in a critical condition necessitating urgent medical treatment, the Idylla EGFR Mutation Assay, with its short turnaround time and low infrastructural requirements, can be considered as a useful technology in pathology laboratories. At present, Idylla EGFR has been validated as a CE-marked IVD product. An overall percentage agreement of 95.9% (95% CI, 91.8%−100%) between the Idylla EGFR Mutation Test and the reference test has been obtained; and the product is able to detect allelic frequencies at ≤5% for mutations in exons 19, 20, and 21 of the *EGFR* oncogene and ≤10% for mutations in exon 18 of the *EGFR* oncogene.

Conclusion

The current multicenter comparative study showed, with the help of a large sample set, that the Idylla EGFR Mutation Assay is a sensitive and highly reliable test to detect the most common and clinically relevant *EGFR* mutations in FFPE tumor tissue, to quickly support the further assessment by the tumor review board along with other genomic, pathologic, and clinical data.

Acknowledgments

We thank Pia Rovsing-Sandholm, Thomas H.L. Jensen (Rigshospitalet, Denmark), Kiki J.H.A. Schouw (Maastricht UMC+), Vitor Sousa, Ana-Filipa Ladeirinha, Lina Carvalho, Ana Alarcão (Institute of Anatomical and Molecular Pathology, University of Coimbra, Coimbra, Portugal), Dieter Techel [Medizinisches Versorgungszentrum (MVZ) Zentrum für Pathologie und Zytodiagnostik GmbH (ZPZ)], Xavier Matias-Guiu Guia, Roger L. Sanuy, Nuria Baixeras-Gonzalez, Rosa Penin-Mosquera (Hospital Universitari de Bellvitge), Jennifer Ridgway, Lynette Chairuka, Michaela Bryce, Tracey Cowey, Rodielyn Brown, Vanessa Peters, Darren Taylor (Canberra Hospital), Piombo Chiara (Humanitas Research Hospital, Milan), Érica Torres (Hospital del MAR, Barcelona), Peter Wild, Susanne Dettwiler, Fabiola Prutek (University Hospital Zurich), Destro Annarita (University Hospital Zurich, Zurich, Switzerland), and Alba Dalmases (Hospital del Mar, Barcelona, Spain) for performing parts of the experiments and analyzing data; Thomas H.L. Jensen (Rigshospitalet, Denmark) for the assessment of all the tissue specimens; Magnus Sundström (Klinisk Patologi, Akademiska Sjukhuset, Uppsala, Sweden) for analyzing the comparative method; and Luc Geeraert for writing the manuscript.

Supplemental Data

Supplemental material for this article can be found at *https://doi.org/10.1016/j.jmoldx.2019.06.010*.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F: Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015, 136:E359−E386
2. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JW, Comber H, Forman D, Bray F: Cancer incidence and

mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer 2013, 49:1374−1403
3. Siegel RL, Miller KD, Jemal A: Cancer statistics, 2017. CA Cancer J Clin 2017, 67:7−30
4. Rahal Z, El Nemr S, Sinjab A, Chami H, Tfayli A, Kadara H: Smoking and lung cancer: a geo-regional perspective. Front Oncol 2017, 7:194
5. Heist RS, Engelman JA: SnapShot: non-small cell lung cancer. Cancer Cell 2012, 21:448.e2
6. Ciardiello F, Tortora G: EGFR antagonists in cancer treatment. N Engl J Med 2008, 358:1160−1174
7. Lee JK, Hahn S, Kim DW, Suh KJ, Keam B, Kim TM, Lee SH, Heo DS: Epidermal growth factor receptor tyrosine kinase inhibitors vs conventional chemotherapy in non-small cell lung cancer harboring wild-type epidermal growth factor receptor: a meta-analysis. JAMA 2014, 311:1430−1437
8. Yarden Y: The EGFR family and its ligands in human cancer: signalling mechanisms and therapeutic opportunities. Eur J Cancer 2001, 37 Suppl 4:S3−S8
9. Linardou H, Dahabreh IJ, Bafaloukos D, Kosmidis P, Murray S: Somatic EGFR mutations and efficacy of tyrosine kinase inhibitors in NSCLC. Nat Rev Clin Oncol 2009, 6:352−366
10. Sequist LV, Waltman BA, Dias-Santagata D, Digumarthy S, Turke AB, Fidias P, Bergethon K, Shaw AT, Gettinger S, Cosper AK, Akhavanfard S, Heist RS, Temel J, Christensen JG, Wain JC, Lynch TJ, Vernovsky K, Mark EJ, Lanuti M, Iafrate AJ, Mino-Kenudson M, Engelman JA: Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. Sci Transl Med 2011, 3:75ra26
11. Hirsch FR, Bunn PA Jr: EGFR testing in lung cancer is ready for prime time. Lancet Oncol 2009, 10:432−433
12. Fang S, Wang Z: EGFR mutations as a prognostic and predictive marker in non-small-cell lung cancer. Drug Des Devel Ther 2014, 8: 1595−1611
13. Li K, Yang M, Liang N, Li S: Determining EGFR-TKI sensitivity of G719X and other uncommon EGFR mutations in non-small cell lung cancer: perplexity and solution (review). Oncol Rep 2017, 37: 1347−1358
14. Dahabreh IJ, Linardou H, Siannis F, Kosmidis P, Bafaloukos D, Murray S: Somatic EGFR mutation and gene copy gain as predictive biomarkers for response to tyrosine kinase inhibitors in non-small cell lung cancer. Clin Cancer Res 2010, 16:291−303
15. Lee CK, Brown C, Gralla RJ, Hirsh V, Thongprasert S, Tsai CM, Tan EH, Ho JC, Chu da T, Zaatar A, Osorio Sanchez JA, Vu VV, Au JS, Inoue A, Lee SM, Gebski V, Yang JC: Impact of EGFR inhibitor in non-small cell lung cancer on progression-free and overall survival: a meta-analysis. J Natl Cancer Inst 2013, 105:595−605
16. Laurie SA, Goss GD: Role of epidermal growth factor receptor inhibitors in epidermal growth factor receptor wild-type non-small-cell lung cancer. J Clin Oncol 2013, 31:1061−1069
17. Yasuda H, Kobayashi S, Costa DB: EGFR exon 20 insertion mutations in non-small-cell lung cancer: preclinical data and clinical implications. Lancet Oncol 2012, 13:e23−e31
18. Novello S, Barlesi F, Califano R, Cufer T, Ekman S, Levra MG, Kerr K, Popat S, Reck M, Senan S, Simo GV, Vansteenkiste J, Peters S: ESMO Guidelines Committee: Metastatic non-small-cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2016, 27 Suppl 5:v1−v27
19. Kalemkerian GP, Narula N, Kennedy EB, Biermann WA, Donington J, Leighl NB, Lew M, Pantelas J, Ramalingam SS, Reck M, Saqi A, Simoff M, Singh N, Sundaram B: Molecular testing guideline for the selection of patients with lung cancer for treatment with targeted tyrosine kinase inhibitors: American Society of Clinical Oncology Endorsement of the College of American Pathologists/International Association for the Study of Lung Cancer/-Association for Molecular Pathology Clinical Practice Guideline Update. J Clin Oncol 2018, 36:911−919
20. Kerr KM, Bubendorf L, Edelman MJ, Marchetti A, Mok T, Novello S, O'Byrne K, Stahel R, Peters S, Felip E; Panel Members: Second ESMO consensus conference on lung cancer: pathology and molecular biomarkers for non-small-cell lung cancer. Ann Oncol 2014, 25:1681−1690
21. NCCN: NCCN Guidelines Version 1.2018. Non-Small Cell Lung Cancer. Plymouth Meeting, PA: National Comprehensive Cancer Network, 2017
22. Jänne PA, Yang JC, Kim DW, Planchard D, Ohe Y, Ramalingam SS, Ahn MJ, Kim SW, Su WC, Horn L, Haggstrom D, Felip E, Kim JH, Frewer P, Cantarini M, Brown KH, Dickinson PA, Ghiorghiu S, Ranson M: AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer. N Engl J Med 2015, 372:1689−1699
23. Ramalingam SS, Yang JC, Lee CK, Kurata T, Kim DW, John T, Nogami N, Ohe Y, Mann H, Rukazenkov Y, Ghiorghiu S, Stetson D, Markovets A, Barrett JC, Thress KS, Jänne PA: Osimertinib as first-line treatment of EGFR mutation-positive advanced non-small-cell lung cancer. J Clin Oncol 2018, 36:841−849
24. Soria JC, Ohe Y, Vansteenkiste J, Reungwetwattana T, Chewaskulyong B, Lee KH, Dechaphunkul A, Imamura F, Nogami N, Kurata T, Okamoto I, Zhou C, Cho BC, Cheng Y, Cho EK, Voon PJ, Planchard D, Su WC, Gray JE, Lee SM, Hodge R, Marotti M, Rukazenkov Y, Ramalingam SS; FLAURA Investigators: Osimertinib in untreated EGFR-mutated advanced non-small-cell lung cancer. N Engl J Med 2018, 378:113−125
25. Thunnissen E, Kerr KM, Herth FJ, Lantuejoul S, Papotti M, Rintoul RC, Rossi G, Skov BG, Weynand B, Bubendorf L, Katrien G, Johansson L, López-Ríos F, Ninane V, Olszewski W, Popper H, Jaume S, Schnabel P, Thiberville L, Laenger F: The challenge of NSCLC diagnosis and predictive analysis on small samples: practical approach of a working group. Lung Cancer 2012, 76:1−18
26. Overman MJ, Modak J, Kopetz S, Murthy R, Yao JC, Hicks ME, Abbruzzese JL, Tam AL: Use of research biopsies in clinical trials: are risks and benefits adequately discussed? J Clin Oncol 2013, 31: 17−22
27. Legras A, Barritault M, Tallet A, Fabre E, Guyard A, Rance B, Digan W, Pecuchet N, Giroux-Leprieur E, Julie C, Jouveshomme S, Duchatelle V, Giraudet V, Gibault L, Cazier A, Pastre J, Le Pimpec-Barthes F, Laurent-Puig P, Blons H: Validity of targeted next-generation sequencing in routine care for identifying clinically relevant molecular profiles in non-small-cell lung cancer: results of a 2-year experience on 1343 samples. J Mol Diagn 2018, 20:550−564
28. Leduc C, Merlio JP, Besse B, Blons H, Debieuvre D, Bringuier PP, Monnet I, Rouquette I, Fraboulet-Moreau S, Lemoine A, Pouessel D, Mosser J, Vaylet F, Langlais A, Missy P, Morin F, Moro-Sibilot D, Cadranel J, Barlesi F, Beau-Faller M; French Cooperative Thoracic Intergroup (IFCT): Clinical and molecular characteristics of non-small-cell lung cancer (NSCLC) harboring EGFR mutation: results of the nationwide French Cooperative Thoracic Intergroup (IFCT) program. Ann Oncol 2017, 28:2715−2724
29. Patton S, Normanno N, Blackhall F, Murray S, Kerr KM, Dietel M, Filipits M, Benlloch S, Popat S, Stahel R, Thunnissen E: Assessing standardization of molecular testing for non-small-cell lung cancer: results of a worldwide external quality assessment (EQA) scheme for EGFR mutation testing. Br J Cancer 2014, 111:413−420
30. Pirker R, Herth FJ, Kerr KM, Filipits M, Taron M, Gandara D, Hirsch FR, Grunenwald D, Popper H, Smit E, Dietel M, Marchetti A, Manegold C, Schirmacher P, Thomas M, Rosell R, Cappuzzo F, Stahel R; European EGFR Workshop Group: Consensus for EGFR mutation testing in non-small cell lung cancer: results from a European workshop. J Thorac Oncol 2010, 5:1706−1713
31. Kerr KM, Dafni U, Schulze K, Thunnissen E, Bubendorf L, Hager H, ETOP Lungscape Consortium, et al: Prevalence and clinical association of gene mutations through multiplex mutation testing in patients with NSCLC: results from the ETOP Lungscape Project. Ann Oncol 2018, 29:200−208

32. Tu HY, Ke EE, Yang JJ, Sun YL, Yan HH, Zheng MY, Bai XY, Wang Z, Su J, Chen ZH, Zhang XC, Dong ZY, Wu SP, Jiang BY, Chen HJ, Wang BC, Xu CR, Zhou Q, Mei P, Luo DL, Zhong WZ, Yang XN, Wu YL: A comprehensive review of uncommon EGFR mutations in patients with non-small cell lung cancer. Lung Cancer 2017, 114:96−102
33. Yi S, Zhuang Y, Zhou J, Ma H, Huang J, Wang L, Zhu W, Kang S, Guo L, Guo F: A comparison of epidermal growth factor receptor mutation testing methods in different tissue types in non-small cell lung cancer. Int J Mol Med 2014, 34:464−474
34. Han Y, Li J: Sample types applied for molecular diagnosis of therapeutic management of advanced non-small cell lung cancer in the precision medicine. Clin Chem Lab Med 2017, 55:1817−1833
35. Park S, Holmes-Tisch AJ, Cho EY, Shim YM, Kim J, Kim HS, Lee J, Park YH, Ahn JS, Park K, Jänne PA, Ahn MJ: Discordance of molecular biomarkers associated with epidermal growth factor receptor pathway between primary tumors and lymph node metastasis in non-small cell lung cancer. J Thorac Oncol 2009, 4:809−815
36. Zhang J, Fujimoto J, Zhang J, Wedge DC, Song X, Zhang J, Seth S, Chow CW, Cao Y, Gumbs C, Gold KA, Kalhor N, Little L, Mahadeshwar H, Moran C, Protopopov A, Sun H, Tang J, Wu X, Ye Y, William WN, Lee JJ, Heymach JV, Hong WK, Swisher S, Wistuba II, Futreal PA: Intratumor heterogeneity in localized lung adenocarcinomas delineated by multiregion sequencing. Science 2014, 346:256−259
37. Chen G, Mosier S, Gocke CD, Lin MT, Eshleman JR: Cytosine deamination is a major cause of baseline noise in next-generation sequencing. Mol Diagn Ther 2014, 18:587−593
38. De Luca C, Rappa AG, Gragnano G, Malapelle U, Troncone G, Barberis M: Idylla assay and next generation sequencing: an integrated EGFR mutational testing algorithm. J Clin Pathol 2018, 71:745−750