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► To cite this version:

Claire Bocciarelli, Justine Cohen, Romain Pelletier, Jeanne Tran van Nhieu, Jonathan Derman, et al.. Evaluation of the Idylla system to detect the EGFRT790M mutation using extracted DNA. Pathology - Research and Practice, 2020, 216 (1), pp.152773. 10.1016/j.prp.2019.152773 . hal-03489789

HAL Id: hal-03489789

<https://hal.science/hal-03489789v1>

Submitted on 21 Jul 2022

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Evaluation of the Idylla system to detect the *EGFR*^{T790M} mutation using extracted DNA

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Disclosure of Potential Conflict of Interest: The authors declare no conflict of interest.

Abstract count number: 261

Manuscript word count number: 2633

Reference number: 12

Figure number: 2

Table number: 3

Supplementary files: 0

ABSTRACT

Introduction: During the last few years, detection of epidermal growth-factor–receptor (*EGFR*)-activating mutations has become a routine part of clinical practice because of their importance in choosing the optimal treatment strategy for non-small-cell lung cancers (NSCLCs). The emergence of third-generation *EGFR*–tyrosine-kinase inhibitors required the implementation of sensitive methods to detect the subclonal *EGFR*^{T790M} mutation. Clinical implications make it essential to rapidly search for the *T790M* mutation, which is a real challenge for laboratories. The aim of this study was to compare performances of next-generation sequencing (NGS), one of the most frequently used molecular biology methods, and Idylla *EGFR*-Mutation Assay (henceforth Idylla), a fully automated real-time polymerase chain reaction (PCR) that is increasingly used in pathology laboratories, to detect the *EGFR*^{T790M} mutation using DNA.

Methods: This retrospective study used 47 DNA samples extracted from NSCLC biopsies that previous NGS identified as: 29 harboring *EGFR* and *T790M* resistance mutations, 11 *EGFR*-activating mutation without *T790M* and 7 wild-type *EGFR*. *EGFR*^{T790M} limit-of-detection (LOD) experiments used a commercial DNA known to harbor that mutation.

Results: Idylla detected primary *EGFR*-activating mutations and the *T790M* mutation in 97.5% and 65.5% of the cases, respectively. The results of this retrospective analysis and LOD experiments showed that the Idylla should only be used to detect *EGFR* mutations in samples with > 25 ng of DNA and > 10% tumor cells.

Conclusions: Idylla was able to rapidly detect *EGFR*-activating mutations but detecting subclone mutations, like *T790M*, with < 25 ng of good-quality DNA or < 10% tumor cells (variant allele frequency below the assay’s validated LOD) was not always reliable.

Keywords:

Idylla

Next-generation sequencing

EGFR^{T790M}

Lung cancer

Extracted DNA

1 Introduction

2

3 Lung cancer is the leading cause of death from cancer worldwide, responsible for ~1.8
4 million deaths in 2018. Non-small-cell lung cancer (NSCLC) is the predominant form of lung
5 cancer [1]. Patients often have advanced disease at diagnosis, which carries a poor prognosis
6 and 5-year overall survival of only 18% [2].

7 The recent development of personalized therapies has considerably improved NSCLC
8 outcomes. In addition to the driver-gene alterations found in NSCLCs, epidermal growth-
9 factor-receptor (*EGFR*) mutations, mostly exon 19 deletions (*Del19*) and exon 21 *L858R*
10 mutation, are the most useful because of their associated sensitivity to *EGFR*-tyrosine-kinase
11 inhibitors (*EGFR*-TKIs) [3]. Therefore, the search for *EGFR*-activating mutations in
12 advanced NSCLCs is now essential to choose the optimal therapeutic strategy. Even though
13 patients with *EGFR* mutations initially respond to *EGFR*-TKIs, a large majority of their
14 tumors ultimately acquire TKI resistance [4]. Several resistance mechanisms to first- or
15 second-generation *EGFR*-TKIs have been described, especially the *EGFR*^{T790M} point mutation
16 in exon 20, which is the most frequently observed. Hence, third-generation *EGFR*-TKIs have
17 been designed to inhibit the gene harboring the *EGFR*-activating mutation and the *T790M*
18 resistance mutation; they have marked clinical benefit for patients whose tumors carry these
19 molecular alterations [5].

20 Detecting *EGFR* mutations in NSCLCs has become a routine part of clinical practice but
21 the implementation of these analyses, especially *T790M* detection, can be challenging for
22 several reasons. First, formalin-fixed paraffin embedded (FFPE) tissue samples are usually
23 small, due to the thinness of tumor biopsies, which limits the amount of DNA available to
24 detect mutated *EGFR*. Second, the *T790M* mutation is usually found in a subclone, with low
25 variant allele frequency (VAF), requiring a highly sensitive method to be detected. Third, the

26 analysis turnaround time must be short, especially for patients with rapid clinical progression.

27 Therefore, the approach used to detect the *EGFR*^{T790M} mutation must be highly sensitive,
28 fast and economically acceptable. Today, next-generation sequencing (NGS) is a frequently
29 used molecular biology method. Although NGS is very sensitive, it requires technical
30 expertise and bioinformatics skills, has long turnaround times and is profitable only for testing
31 large case volumes. Recently, other techniques have been developed to detect *EGFR*
32 mutations, like the Idylla™ *EGFR*-Mutation Assay (henceforth, Idylla) (Biocartis NV,
33 Mechelen, Belgium), that is a fully automated real-time polymerase chain reaction (PCR;
34 instrument plus software), able to provide rapid *EGFR*-genotyping. It can analyze FFPE tissue
35 sections or extracted DNA. Little molecular biology expertise is required, results can be
36 obtained within 150 minutes and hands-on time is minimal. For these reasons, this system is
37 increasingly used in pathology laboratories to determine *EGFR* status quickly.

38 Recent studies showed Idylla system efficiency at detecting *EGFR* mutations, compared to
39 either NGS or pyrosequencing [6,7], but none of those studies focused on *EGFR*^{T790M}
40 detection, which represents a real challenge as stated above.

41 The aims of this study were to compare the NGS and Idylla abilities to detect the
42 *EGFR*^{T790M} mutation using DNA, to determine the Idylla limit of detection (LOD) for the
43 T790M mutation, and evaluate the impacts of the quality and quantity of DNA on the
44 system's performance.

45

46 **Materials and Methods**

47

48 *Sample selection*

49 This retrospective study was designed to assess the relative performances of NGS and
50 Idylla to detect the *EGFR*^{T790M} mutation. To do so, 47 NSCLC biopsies were selected. They

51 had previously undergone NGS in our Department of Pathology between 2015 and 2018 for
52 molecular diagnosis, in compliance with French regulations; 29 harbored an *EGFR*-activating
53 mutation and the *T790M* resistance mutation, 11 harbored an *EGFR*-activating mutation
54 without *T790M* and 7 were wild-type. The prerequisite for sample selection was the
55 availability of at least 10 µL of residual archival extracted DNA. Each sample had previously
56 undergone routine PCR high-resolution melting (HRM) analysis, targeting exon 2 of the
57 *KRAS* (Kirsten rat-sarcoma viral oncogene) to determine DNA quality, and was accorded a
58 grade of A–D, with A being the best, based on the crossing threshold (Ct) observed during
59 that PCR.

60 For LOD assessment, 100 ng of commercial DNA harboring the *EGFR*^{T790M} mutation with
61 VAF = 50% was used (*EGFR T790M* Reference Standard, Horizon, Horizon Discovery,
62 Cambridge, U.K.). That DNA was serially diluted in control wild-type DNA to obtain a 6-
63 point VAF scale: 50%, 25%, 12.5%, 6.2%, 3.1% and 1.5%.

64

65 *DNA extraction and quantification with a quantitative PCR*

66 All DNA samples had been extracted, after macrodissection when necessary, from FFPE
67 tissue sections (usually 7 sections, 5-µm thick) using the Maxwell 16 FFPE Plus LEV DNA
68 Purification Kit IVD (Promega, Charbonnières-les-Bains, France), according to the
69 manufacturer's instructions. The DNA was quantified using a Qubit fluorimeter in
70 combination with the Qubit dsDNA HS Array Kit (ThermoFisher Scientific, Waltham, MA,
71 U.S.A.).

72

73 *NGS*

74 For NGS, 10 ng of DNA (as measured by fluorimetry) was amplified using the Ion
75 AmpliSeq™ Colon and Lung Cancer Panel (ThermoFisher Scientific), which is a multiplex

76 PCR-based library-preparation method by which many regions (70–150 bp) that encompass
77 many mutational hotspots including *EGFR*^{T790M} are amplified. Amplicons were then digested,
78 barcoded and amplified by using the Ion OncoPrint™ Solid Tumor DNA Kit and Ion Select
79 Barcode Adapter Kit (ThermoFisher Scientific), according to the manufacturer’s instructions.
80 After DNA quantification, 25 pM of each library was multiplexed and clonally amplified on
81 ion-sphere particles (ISP) by emulsion PCR performed on Ion Chef (ThermoFisher
82 Scientific), according to the manufacturer’s instructions. The ISP templates were loaded onto
83 an Ion-520 chip and sequenced on a S5 sequencer with the Ion 510™ & Ion 520™ & Ion
84 530™ Kit–Chef, according to the manufacturer’s instructions. Run performance was assessed
85 and for data analyzed with the Torrent Suite Software v.5.10.0 (ThermoFisher Scientific).
86 Single-nucleotide variants and small indels were detected using the Variant Caller plug-in
87 version 5.10.0.18 with low stringency settings (threshold: 2%). The Integrative Genomics
88 Viewer (IGV v 5.01; Broad Institute, Cambridge, MA, U.S.A.) was used for visual inspection
89 of the aligned reads.

90

91 *Idylla EGFR-mutation test*

92 Each sample was retested using Idylla: 10 µL of the original sample-DNA preparation was
93 directly deposited into the Idylla cartridge. DNA was transported via microfluidic channels in
94 the cartridge into 5 separate PCR chambers, which contained lyophilized PCR reagents (i.e.,
95 primers, probes, enzymes) designed for the qualitative detection of 18 genetic-change types
96 and for which 51 different mutations have been validated. After a 150-minute run, final
97 reports were directly available on the Idylla console and the results presented on screen as
98 either “no mutation detected” or “*EGFR* mutation detected”. An internal Ct value for each
99 sample is provided and indicates the quantity and quality of each DNA sample tested. Results
100 were compared to those previously obtained with NGS.

101

102 **Results**

103

104 *Evaluation of Idylla's ability to detect T790M*

105 Idylla is CE-IVD validated by the manufacturer for FFPE tissue sections but the results of
106 several studies showed that it can be used with extracted DNA directly deposited into the
107 cartridge [6,8]. Pertinently, lung biopsies are rapidly exhausted and it is important to store the
108 material to search for all biomarkers assuring better therapeutic management of the patient.
109 When the patient's tumor does not harbor an *EGFR* mutation, Idylla using extracted DNA
110 allows that DNA to be recovered and reused to run all other the analyses necessary to manage
111 NSCLC patients.

112 In our series, 29 samples carried the exon-20 *T790M* mutation, associated with primary
113 *EGFR*-activating mutation(s), such as: *Del19* for 17 (58.6%), exon-21 *L858R* mutation for 11
114 (37.9%) or exon-18 *G719A* mutation for 1 (3.4%) (Table 1). Eleven samples harbored an
115 *EGFR*-activating mutation without *T790M*: 2 (18%) *L858R* mutations, 2 (18%) *L861Q*
116 mutations, 4 *Del19* (36%) and 3 (27%) *G719A* mutations, one of which was associated with
117 an *S768I* mutation. Seven samples expressed wild-type *EGFR*. An average of 210 ng of DNA
118 were deposited in the cartridge and those samples contained an average of 37.8% tumor cells.
119 NGS results yielded average VAFs of 35% for primary *EGFR*-activating mutation and 16.5%
120 for the *T790M* mutation.

121 Idylla sensitivity to detect *EGFR*-activating mutations was 97.5% (39/40) but fell to 65.5%
122 (19/29) for the detection of *T790M*. Its respective specificities for both mutations were 100%.

123 For samples 3, 10, 23 and 29, Idylla's failure to detect the *T790M* mutation can be
124 explained by the poor quality and/or insufficient quantity of the DNA used for the test. For
125 those samples, the internal Ct values provided by the Idylla always exceeded 25, indicating

126 the higher number of PCR cycles needed to reach detectable DNA, which can be expected
127 with low DNA input or poor-quality DNA. For samples 1, 8 and 14, Idylla failure was
128 probably attributable to the low *T790M*-mutation VAF, because NGS analysis yielded a VAF
129 < 5%. For the remaining samples 5, 22 and 24, neither the amount of DNA used for the test
130 nor the *T790M*-mutation VAF can explain the failure to detect the *T790M* mutation.
131 Nevertheless, the quality-control internal Ct value obtained for total *EGFR* for samples 22 and
132 24, which exceeded 25, reflect the poorer quality of those DNA samples used for the test,
133 which could affect assay performance. Sample 5's Ct value was 24.2 and no explanation was
134 found to explain Idylla's failure to detect the *T790M* mutation.

135 To try to understand this failure, we examined the Idylla Explorer curves for case 5 and
136 observed that a *T790M*-mutation amplification curve was there but it did not pass the system's
137 filter threshold; (Fig. 1). Thus, using Idylla Explorer could help detect the *T790M* mutation
138 and avoid false-negative results.

139 These results highlighted the need for a procedure to avoid false-negative assessment of
140 the *T790M* mutation, especially in samples with low amounts of DNA or DNA samples
141 extracted from tissues containing < 10% tumor cells. Thus, we decided to determine the Idylla
142 LOD for *T790M* and the minimal DNA amount that had to be deposited into the cartridge to
143 avoid false-negative results.

144

145 *Determination of the Idylla LOD for T790M using extracted DNA*

146 In light of the results obtained in retrospective series, our second study objective was to
147 evaluate the Idylla LOD for the *T790M* mutation using a commercial mutated DNA known to
148 harbor the that mutation with VAF = 50%. LOD was measured using 100 ng of the
149 commercial DNA, serially diluted in control wild-type DNA to obtain a 6-point VAF scale:
150 50%, 25%, 12.5%, 6.2%, 3.1% and 1.5%. The *T790M* mutation was detected in samples with

151 VAF > 3.1%. For samples with VAFS = 3.1% and 1.5%, Idylla did not detect the mutation
152 and they were reported to be wild-type (not *EGFR*-mutated) (Table 2). Thus, the *T790M*-
153 assay LOD was estimated to be between 3% and 6% using 100 ng of this commercial DNA
154 lot.

155

156 *Determination of minimum DNA quantity required to detect low-VAF T790M*

157 The third study objective was to assess the impact of the quantity of DNA used in Idylla to
158 detect the *T790M* mutation. We used the same commercial DNA at 3 different VAFs (20%,
159 10% and 5%) serially diluted to obtain a 4-point concentration scale (10,5, 2.5 and 1.25
160 ng/μL). Idylla did not detect the *T790M* mutation in a sample with VAF = 5% and 1.25 ng/μL
161 of DNA, corresponding to deposition of 12.5 ng of DNA into the cartridge (Table 3).

162

163 **Discussion**

164

165 This study was undertaken to evaluate Idylla's ability to detect the *EGFR*^{*T790M*} mutation
166 **using extracted DNA**. The study included a total of 47 NSCLC biopsy samples previously
167 subjected to NGS: 29 harboring an *EGFR*-activating mutation and the *T790M* mutation and
168 11 harboring an *EGFR*-activating mutation without the resistance mutation, and 7 wild type
169 for *EGFR*. In addition, LOD experiments aimed to determine this method's sensitivity for
170 extracted DNA.

171 Idylla results confirmed almost all known *EGFR*-activating mutations (39/40) but failed to
172 detect the *T790M* resistance mutation in about one-third of the samples (10/29). Our LOD
173 findings could explain those failures. First, our experiments showed that the amount of DNA
174 loaded into the cartridge was decisive. Herein, the failure to detect the *T790M* mutation in 4
175 samples could be explained by their low DNA concentrations (< 2 ng/μL). We also

176 demonstrated that LOD ranged from 3% to 6% for the *T790M* mutation using a commercial
177 DNA sample. We also assessed the LOD for the *EGFR*-activating mutation *L858R* and *Del19*,
178 and found them to be, respectively, between 3% and 6% and 1% and 3% (data not shown). In
179 our series, Idylla failed to detect 3 *T790M* mutations because of very low VAFs (1.3–4%) that
180 were well below the manufacturer’s recommended assay LOD. That failure was also reported
181 recently by Evrard et al., who described Idylla’s inability to detect *T790M* mutations in
182 samples with VAFs < 5% [9].

183 Finally, we found the internal Ct value to be a very good marker to check the reliability of
184 the result. Indeed, in our opinion, because of the risk of a false-negative finding, the analysis
185 must be considered non-interpretable for a negative result obtained with a sample having a Ct
186 > 25. Therefore, we propose a decision-tree algorithm to avoid the risk of an Idylla false-
187 negative *T790M*-mutation result (Fig. 2). First, all cases with < 25 ng of DNA or < 10% tumor
188 cells should not be analyzed with Idylla. Second, our experience showed that all the negative
189 findings for samples with Ct > 25 should be retested with another method. In addition, our
190 results showed that using Idylla Explorer, which provides the PCR-amplification curves,
191 could detect mutations that do not pass the filter threshold set by the manufacturer. For the 10
192 discordant samples of our series, only samples 2 and 5 had *T790M*-amplification curves.
193 Thus, despite using this tool, the risk of false-negatives persists.

194 Idylla performance to detect *EGFR* mutations has been evaluated in other studies. De Luca
195 et al. tested 3 *T790M*-mutation–harboring samples identified by NGS and Idylla found all 3
196 [6]. However, they did not clearly state the amounts of DNA they used in the test but their
197 VAFs were $\geq 5\%$. Lambros et al. also retested 4 *T790M*-harboring samples identified by
198 NGS. Idylla detected the *T790M* mutation in one surgical sample but failed to do so in the 3
199 biopsies [10]. Unfortunately, neither the VAFs of the *T790M* mutations not detected by Idylla
200 system nor the amounts of DNA used were given because the assay was run using FFPE

201 slides inserted directly into the cartridge. Nevertheless, their results are in accordance with
202 ours and highlight the need to have a strong algorithm to avoid false-negative *T790M*-
203 mutation findings.

204 Our study results and those previously reported showed that it is preferable to analyze
205 samples with < 10% tumor cells or insufficient DNA quality with other, more sensitive
206 techniques, like NGS, or digital or allele-specific PCR. Their LODs for the search for *EGFR*
207 mutations are comprised between 1% and 5% [11,12]. Nonetheless, it is important to keep in
208 mind that those methods, especially NGS analysis, require much longer times and greater
209 expertise in molecular biology than Idylla.

210 To conclude, our results showed that the Idylla is a rapid and valid option to detect *EGFR*-
211 activating mutations. They, like those reported by Evrard et al. [9] and Gilson et al. [8],
212 demonstrated that Idylla provides adequate findings when run on extracted DNA, which
213 allows specimen storage. The real advantage of this technology is its rapidity because the
214 *EGFR* analysis must have a short turnaround time because of its critical clinical implications.
215 This test should not be used with insufficient (< 25 ng) DNA or < 10% tumor cells, which is
216 often the case for thin lung biopsies. That cautionary approach is particularly true for the
217 detection of subclone mutations, like *T790M*. Applying the algorithm proposed herein could
218 help limit false-negative results.

219

220 **Acknowledgments**

221

222 We thank the molecular biology technicians of the Department of Pathology for their
223 technical participation: Déborah Siroli-Sarda, Céline Bourdaudhui, Caroline Taou and Soraya
224 Mehdaoui. We thank the Biocartis employees who helped conduct this study, especially
225 Cyriel Naud and David Favy.

226

227 **Funding**

228

229 This research did not receive any specific support from funding agencies in the public,
230 commercial or not-for-profit sector.

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264

265 **Table 1**266 Characteristics of the 47 case samples assessed in the Idylla-performance study to detect *EGFR* and its resistance mutations *T790M* vs NGS.

Case	Macro- dissection	% Tumor Cells*	DNA			NGS (Ion Torrent S5)	<i>EGFR</i> -activating mutation VAF	<i>T790M</i> VAF	Idylla		NGS-Idylla concordance
			ng/μL	Quality**	Input				Result	Ct	
1	No	20	6	A	60	<i>L858R + T790M</i>	21%	4%	<i>L858R</i>	25.1	No, <i>T790M</i> not detected
2	No	70	133	A	1330	<i>Del19 + T790M</i>	37%	9%	<i>Del19 + T790M</i>	10	Yes
3	No	20	0.1	NE	1	<i>Del19 + T790M</i>	25%	6%	<i>Del19</i>	27.9	No, <i>T790M</i> not detected
4	Yes	70	12	A	120	<i>T790M + T790M</i>	49%	39%	<i>L858R + T790M</i>	22.7	Yes
5	Yes	40	6	A	60	<i>Del19 + T790M</i>	59%	10%	<i>Del19</i>	24.2	No, <i>T790M</i> not detected
6	Yes	55	3	NE	30	<i>L858R + T790M</i>	95%	10%	<i>L858R + T790M</i>	26.2	Yes
7	Yes	25	4	NE	40	<i>L858R + T790M</i>	36%	36%	<i>L858R + T790M</i>	26.1	Yes
8	No	15	5	B	50	<i>L858R + T790M</i>	2%	1.30%	<i>L858R</i>	27.2	No, <i>T790M</i> not detected
9	No	30	5	B	50	<i>Del19 + T790M</i>	32%	8%	<i>Del19 + T790M</i>	27.2	Yes
10	No	25	2	NE	20	<i>Del19 + T790M</i>	19%	7%	<i>Del19</i>	25.4	No, <i>T790M</i> not detected
11	Yes	40	7	A	70	<i>Del19 + T790M</i>	36%	11%	<i>Del19 + T790M</i>	23.8	Yes
12	Yes	25	12	A	120	<i>Del19 + T790M</i>	40%	19%	<i>Del19 + T790M</i>	23.8	Yes
13	Yes	30	3	NE	30	<i>Del19 + T790M</i>	37%	4%	<i>Del19 + T790M</i>	22.2	Yes
14	Yes	55	4	A	40	<i>L858R + T790M</i>	14%	2%	<i>L858R</i>	24.1	No, <i>T790M</i> not detected
15	Yes	15	2.6	NE	26	<i>Del19 + T790M</i>	42%	21%	<i>Del19 + T790M</i>	25.2	Yes

16	No	40	3	NE	30	<i>L858R + T790M</i>	60%	21%	<i>L858R + T790M</i>	25.9	Yes
17	No	60	111	B	1110	<i>Del19 + T790M</i>	54%	19%	<i>Del19 + T790M</i>	20.1	Yes
18	No	60	75	B	750	<i>Del19 + T790M</i>	18%	10%	<i>Del19 + T790M</i>	21.8	Yes
19	Yes	60	9	B	90	<i>L858R + T790M</i>	25%	34%	<i>L858R + T790M</i>	26.7	Yes
20	Yes	60	12	B	120	<i>Del19 + T790M</i>	21%	13%	<i>Del19 + T790M</i>	26.7	Yes
21	No	60	33	B	330	<i>Del19 + T790M</i>	55%	22%	<i>Del19 + T790M</i>	21.7	Yes
22	Yes	40	28	C	280	<i>L858R + T790M</i>	12%	10%	<i>L858R</i>	27.7	No, <i>T790M</i> not detected
23	Yes	30	1	NE	10	<i>L858R + T790M</i>	13%	3%	Wild type	29.4	No, <i>L858R</i> & <i>T790M</i> not detected
24	Yes	20	8	A	80	<i>Del19 + T790M</i>	17%	6%	<i>Del19</i>	25.4	No, <i>T790M</i> not detected
25	No	10	30	A	300	<i>Del19 + T790M</i>	9%	3%	<i>Del19 + T790M</i>	21.6	Yes
26	No	40	4	NE	40	<i>G719A + T790M</i>	69%	67%	<i>G719A + T790M</i>	24.5	Yes
27	No	35	10	A	100	<i>Del19 + T790M</i>	83%	15%	<i>Del19 + T790M</i>	20.9	Yes
28	No	50	15	A	150	<i>Del19 + T790M</i>	92%	47%	<i>Del19 + T790M</i>	18.8	Yes
29	No	20	1.5	NE	15	<i>L858R + T790M</i>	28%	21%	<i>L858R</i>	26.7	No, <i>T790M</i> not detected
30	Yes	10	16	A	160	<i>L858R</i>	5%	NA	<i>L858R</i>	22	Yes
31	Yes	25	35	A	350	<i>L858R</i>	29%	NA	<i>L858R</i>	20.7	Yes
32	Yes	30	13	B	130	Wild type	NA	NA	Wild type	24.8	Yes
33	No	60	9	A	90	Wild type	NA	NA	Wild type	24.5	Yes

34	Yes	10	16	A	160	Wild type	NA	NA	Wild type	24.8	Yes
35	No	45	11	A	110	<i>L861Q</i>	77%	NA	<i>L861Q</i>	21.7	Yes
36	v	25	42	A	420	<i>Del19</i>	24%	NA	<i>Del19</i>	20.1	Yes
37	No	55	34	B	340	<i>G719A</i>	37%	NA	<i>G719A</i>	22.4	Yes
38	Yes	30	11	A	110	<i>G719A</i>	13%	NA	<i>G719A</i>	23.6	Yes
39	No	20	8	B	80	<i>Del19</i>	NA	NA	<i>Del19</i>	25.4	Yes
40	No	60	4	NE	40	Wild type	NA	NA	Wild type	25.9	Yes
41	No	40	13	B	130	Wild type	NA	NA	Wild type	23	Yes
42	Yes	30	17	B	170	<i>G719A + S768I</i>	22%	NA	<i>G719A + S768I</i>	23.1	Yes
43	No	60	85	A	850	<i>L861Q</i>	20%	NA	<i>L861Q</i>	19.1	Yes
44	No	65	13	B	130	Wild type	NA	NA	Wild type	24.5	Yes
45	Yes	40	32.3	B	323	<i>Del19</i>	22%	NA	<i>Del19</i>	22	Yes
46	No	50	60	A	600	<i>Del19</i>	20%	NA	<i>Del19</i>	19.2	Yes
47	No	5	26	A	260	Wild type	NA	NA	Wild type	20.7	Yes

267 *EGFR*, epidermal growth-factor-receptor; NGS, next-generation sequencing; VAF, variant allele frequency; Ct, crossing threshold; *Del19*, exon 19 deletion; NA, not applicable

268 * the percent tumor cells indicated corresponds to that obtained after macrodissection **Quality graded from A to D, with A being the best; NE, not evaluated.

269 **Table 2**

270 Idylla limits of detection obtained with a commercial DNA harboring the *EGFR*^{T790M} mutation.

<i>T790M</i> VAF, %	DNA loaded		<i>EGFR</i> Ct control	Idylla result
	Volume, μ L	Quantity, ng		
50	10	100	23.4	Mutated
25	10	100	23.2	Mutated
12.5	10	100	23.1	Mutated
6.3	10	100	23.3	Mutated
3.1	10	100	23.1	Wild type
1.6	10	100	22.7	Wild type

271 *EGFR*, epidermal growth-factor–receptor; VAF, variant allele frequency; Ct, crossing threshold.

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294 **Table 3**

295 Determination of the minimal amount of DNA required in the *EGFR* Idylla assay.

<i>T790M</i>	DNA loaded			<i>EGFR</i>	Idylla
	VAF, %	ng	Volume, μ L		
20	10	10	100	22.1	Mutated
20	5	10	50	23.2	Mutated
20	2.5	10	25	24.2	Mutated
20	1.25	10	12.5	24.8	Mutated
10	10	10	100	22.0	Mutated
10	5	10	50	23.6	Mutated
10	2.5	10	25	24.3	Mutated
10	1.25	10	12.5	26.3	Mutated
5	10	10	100	22.2	Mutated
5	5	10	50	23.3	Mutated
5	2.5	10	25	24.3	Mutated
5	1.25	10	12.5	25.8	Wild type

296 *EGFR*, epidermal growth-factor–receptor; VAF, variant allele frequency; Ct, crossing threshold.

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299 **FIGURE Legends**

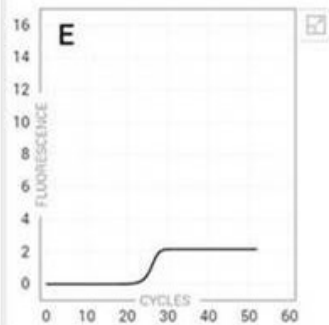
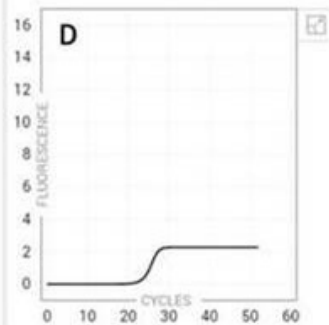
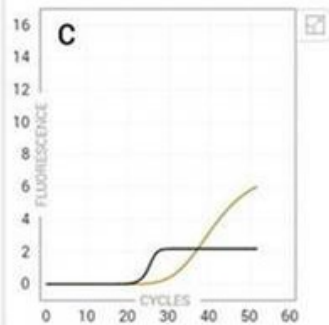
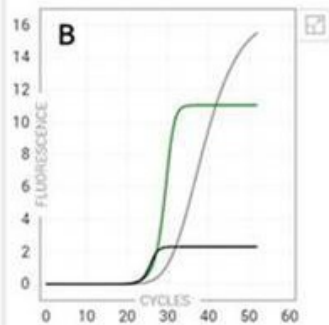
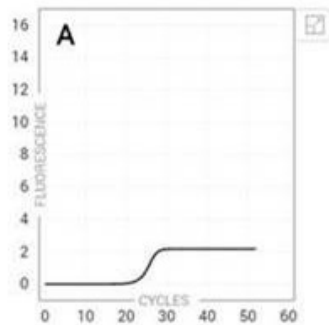
300

301 **Fig. 1.** Idylla Explorer printout of real-time PCR curves the showing the method's failure to detect the
302 epidermal growth-factor–receptor (*EGFR*) *T790M* mutation in sample 5, despite having loaded
303 sufficient DNA into the cartridge, an adequate percentage of tumor cells and good internal crossing
304 threshold (**CQ on the printout**). Total *EGFR*-control DNA was successfully amplified (**black curves,**
305 A–E, with A being the best) with CQ = 24.1. B. An exon 19 deletion (*Del19*) mutation is detected
306 (**green curve**) with CQ = 27.8 (Δ CQ = 3.4) and a *T790M*-mutation amplification curve (**yellow curve**),
307 with CQ = 35.9, which failed to pass the system's filter threshold (Δ CQ = 11.7).

308 **Fig. 2.** Algorithm proposed to avoid epidermal growth-factor–receptor (*EGFR*) false-negative *T790M*-
309 mutation detection when using Idylla. All samples containing < 25 ng of DNA or < 10% tumor cells
310 should **not** be analyzed with the Idylla. All Idylla-determined *T790M*-negative samples with a crossing
311 threshold (Ct) > 25 should be retested with another method, like next-generation sequencing (NGS).

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***** **EGFR GENOTYPE:**
EXON 19 DELETION: MUTATION DETECTED

[Run details](#)

TARGET	CQ	Δ CQ
EGFR Total	24.16	-

TARGET	CQ	Δ CQ
Del 15	27.84	3.41
Del 9/21a	34.33	9.89
EGFR Total	24.44	-

TARGET	CQ	Δ CQ
T790M	35.97	11.77
EGFR Total	24.20	-

TARGET	CQ	Δ CQ
EGFR Total	24.40	-

TARGET	CQ	Δ CQ
EGFR Total	24.88	-

