ORIGINAL ARTICLE

Ultrarapid *EGFR* Mutation Screening Followed by Comprehensive Next-Generation Sequencing: A Feasible, Informative Approach for Lung Carcinoma Cytology Specimens With a High Success Rate

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ABSTRACT

Introduction: For patients with advanced NSCLC, cytologic samples may be the only diagnostic specimen available for molecular profiling. Although both rapid and comprehensive assessment are essential in this setting, an integrated multitest approach remains an important strategy in many laboratories, despite the risks and challenges when working with scant samples. In this study, we describe our experience and high success rate in using a multitest approach, focusing on the clinical validation and incorporation of ultrarapid *EGFR* testing using the Idylla system followed by comprehensive next-generation sequencing (NGS).

Methods: Cytology samples received for routine molecular testing were included in this study. The performance characteristics of the *EGFR* Idylla assay were assessed; tissue suitability parameters and interpretation criteria to supplement automated mutation calling were established. The assay performance was monitored for 1 year, comparing the results with those of concurrent NGS testing by MSK-IMPACT (primarily) or MSK-AmpliSeq and MSK-Fusion solid panel in a subset of cases.

Results: Overall, 301 samples were studied; 83 samples were included in validation (60.2% [50 of 83] were positive for *EGFR* mutations). Concordance with the reference method was 96.4% (80 of 83) of the samples with excellent reproducibility. The limit of detection was variable depending on the total tissue input and the specific mutation tested. Unextracted tissue inputs that maintained total *EGFR* cycle of quantification at less than 23 allowed all mutations to be detected if present at greater than 5% variant allele frequency. Mutations could be detected at 1% variant allele frequency with total *EGFR* cycle of quantification of 18. During the clinical implementation phase, 218 NSCLC samples were tested by Idylla (24.3% [53 of 218] were *EGFR* mutation positive). Concurrent NGS testing was requested on 165 samples and successfully performed on 96.4% (159 of 165) of the samples. The Idylla automated results were concordant with those obtained by NGS in 96.2% (153 of 159) of cases and improved to 98.7% (157 of 159) after incorporation of manual review criteria to supplement automated calling, resulting in a diagnostic sensitivity of 95.6% (95% confidence interval: 84.9%–99.5%). In general, 9% (14 of 159) of the cases tested by NGS had *EGFR* mutations not covered by the Idylla assay, primarily

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insertions in exon 19 and 20 and minor mutations co-occurring with canonical sensitizing mutations.

Conclusions: Comprehensive molecular testing is feasible and has a high success rate in NSCLC cytology samples when using a multitest approach. Testing with the Idylla system enables rapid and accurate determination of the *EGFR* status without compromising subsequent NGS testing.

Keywords: Molecular profiling of lung cancer; Ultrarapid EGFR testing; Next-generation sequencing in small samples; EGFR testing; Cytology molecular

Introduction

Detection of actionable genetic biomarkers has become the standard of care for therapy selection and clinical management in patients with advanced NSCLC. Despite the guidelines[1–5] and broad agreement on the importance of biomarker testing, many patients continue to go untested or begin nontargeted treatment before test results become available. This occurs even for canonical targetable mutations in *EGFR,* which have been the standard of practice since 2011.[6] Barriers to testing identified by US and global surveys include insufficient tissue for testing, relatively long turnaround times (TATs), lack of local molecular testing, and poor performance status of patients.[7–18]

Given the growing number of actionable targets in NSCLC, current guidelines[2] support the position that upfront comprehensive next-generation sequencing (NGS) is more practical than performing a series of single-gene assays. Recent advances in checkpoint immunotherapy further underscore the need for more comprehensive NGS testing to accurately assess new markers, such as tumor mutation burden, mutational signatures, and microsatellite instability.[19] However, translating these rapidly evolving needs into routine clinical practice presents many logistical challenges. Aside from the complexity of adopting the NGS technology in clinical laboratories, TATs for comprehensive NGS assays may be several weeks and are, therefore, unsuitable for timely selection of first-line treatment. In addition, given the broad range of markers, a single DNA-based NGS assay may not be able to assess all biomarkers.[20]

Cytology specimens are often the sole source of tumor tissue for molecular profiling in patients with NSCLC, in view of the increased emphasis on minimally invasive procedures to obtain diagnostic material. Although studies have demonstrated the suitability of these samples with limited tissue for molecular testing,[21–26] including comprehensive NGS,[27] success rates vary widely across institutions and are lower when a sample is subjected to serial single-gene testing instead of an upfront multiplexed assay.[21,28–30] Improvement in success rates relies on a careful choice of technology and optimization of tissue handling and testing protocols. In this work, we describe an integrated approach to molecular testing for NSCLC cytology samples. Our approach incorporates upfront immunohistochemistry for surrogate fusion screening, rapid assessment of *EGFR* mutations using the Idylla platform, and comprehensive DNA and RNA NGS. This study focuses on the validation of the Idylla assay as a critical step to minimize tissue utilization for several types of cytology preparations, including both extracted and unextracted material from formalin-fixed paraffin-embedded (FFPE) tissue blocks, supernatants or minimal residual aspirate material in CytoLyt, and stained smears. A combined approach using aliquots of the precapture NGS libraries for rapid *EGFR* testing is also described for specimens with minimal amount of material. Finally, the results of concurrent NGS testing are compared and discussed.

Materials and Methods

Case Selection and Sample Preparation

Cytology samples with a confirmed diagnosis of lung adenocarcinoma or NSCLC favoring adenocarcinoma (on the basis of morphology and immunohistochemistry) and submitted for routine molecular testing at the Memorial Sloan Kettering Cancer Center were assessed in this study. For the validation phase, a set of archival samples with known *EGFR* mutation status (50 positive and 33 negative) was selected, representing several types of cytology preparations, including sections from FFPE cell blocks (3–5 unstained tissue sections, 5 μm thick), residual material in CytoLyt, tissue recovered from Diff-Quick stained aspirate smears, extracted genomic DNA, and aliquots of precapture libraries previously tested by MSK-IMPACT,[31] our hybridization capture-based NGS assay for targeted deep sequencing of all exons and selected introns of 468 cancer genes. Primary mutation analysis was performed using one of the following three reference methods on the basis of previously described protocols: fragment analysis[32] for detection of indels in exons 19 and 20 of *EGFR*; MSK-IMPACT[31] or MSK-AmpliSeq,[33] and an amplicon-based NGS assay for interrogation of targeted regions in 96 cancer genes in specimens with very scant tissue.

For the clinical implementation phase, all cytology cases submitted for routine molecular testing at the Memorial Sloan Kettering Cancer Center between September 13, 2018, and November 11, 2019, were identified. The stained slides were reviewed to visually assess tumor content. For FFPE cell-block specimens, 20 (5-μm thick) unstained slides were obtained per case, in which three to five were retained for Idylla testing and 15 to 17 were submitted for DNA extraction in anticipation of subsequent NGS. The samples of residual cytology material in CytoLyt were prepared as previously described[21]; 20 μL aliquots of the unextracted material were used for Idylla, and the remaining material was extracted for NGS. The stained smears were scraped and processed without destaining as previously detailed.[34]

EGFR Testing by the Idylla System

Rapid *EGFR* testing was performed using single-use cartridges on the Idylla platform according to the manufacturer's protocol.[35,36] In brief, the Idylla *EGFR* mutation assay is an automated, cartridge-based assay that assesses 51 mutations (Supplementary Table 1). The cartridge is an enclosed system with a dedicated chamber for direct tissue input without the need for previous DNA extraction. Tissue lysis and nucleic acid amplification occur within the cartridge. Microfluidic channels then transport the nucleic acids into five separate chambers containing predeposited dried polymerase chain reaction (PCR) reagents. Testing for specific variants in each chamber is performed by real-time PCR with a fluorophore-based detection system. Six fluorophores are incorporated per chamber, enabling the simultaneous analysis of mutations and an endogenous sample processing control (total *EGFR*). Fluorescence data are analyzed using the Test Type Package software version 1.2, which is translated into genetic variant calls on the basis of a proprietary analysis pipeline. The PCR amplification curves can be visualized through a web-based interface, Idylla Explore, that provides information on sample metrics, including cycle of quantification (Cq) for total and mutant *EGFR* and delta Cq (difference between the Cq of mutant *EGFR* and total *EGFR* from the same chamber [ΔCq]).

Validation of the Idylla Assay

The accuracy, diagnostic sensitivity, analytical sensitivity, minimum input requirements, and reproducibility of the Idylla *EGFR* assay were evaluated. To assess analytical sensitivity and the limit of detection, dilution studies were performed on extracted DNA to establish the minimum internal quality metrics required for reliable detection of specific *EGFR* mutations. A mixed positive control sample was prepared from the H1650 cell line (positive for exon 19 deletion) and the H1975 cell line (positive for both *EGFR* exon 21 L858R and T790M mutations), with the three mutations present at variant allele frequencies (VAFs) of approximately 24%, 35%, and 32%, respectively. The mixed control was then diluted with *EGFR* wild-type DNA from the H1781 cell line at 50%, 25%, 12.5%, 6.25%, 3.13%, and 1.56% dilutions (Supplementary Table 2). Studies to assess minimum input requirements for variant detection at 5% and 15% VAF were also performed using commercial controls HD777 and HD730 at inputs of 100, 50, 25, 10, and 5 ng of DNA (Supplementary Table 3). Finally, interassay reproducibility was assessed using six patient samples (three positive and three negative) tested three times on different instruments and a control sample (HD730) tested 32 times across eight instruments.

Clinical Implementation of the Assay and Comparison With NGS Testing

After the initial validation, the clinical performance of the assay was assessed for 1 year. Idylla *EGFR* testing was performed as a reflex test on all confirmed lung adenocarcinoma cases. Concurrent MSK-IMPACT was performed on clinician request if the patient provided informed consent. Cases with insufficient material for MSK-IMPACT were reflexed to the targeted MSK-AmpliSeq panel (if requested). Cases without identifiable mitogenic drivers or those requiring confirmation of structural variants detected by MSK-IMPACT were reflexed to the MSK-Fusion solid panel, our custom clinical gene-fusion detection assay for solid tumors that utilizes ArcherDx Anchored Multiplex PCR (AMP) technology for targeted RNA sequencing.[20]

Results

Validation of the Assay

Overall, 83 clinical samples were included in the Idylla *EGFR* validation study, encompassing both extracted and unextracted materials: 14 unextracted FFPE unstained slides, 13 unextracted Diff-Quick stained slides, 11 DNA from FFPE, 14 DNA from cell pellet, 15 unextracted supernatant or residual cytology specimens in CytoLyt, and 16 precapture NGS libraries. Among these, 60.2% (50 of 83) were positive for one or more mutations in *EGFR* (68 mutations). The results are summarized in Figures 1 and 2 and Supplementary Table 4.

The mutations in *EGFR* were present at VAFs ranging from 2.6% to 83.7% on the basis of concurrent NGS analysis. Using the Idylla software for mutation calling, concordant results were obtained in 96.4% (80 of 83) of cases as compared with the reference method,

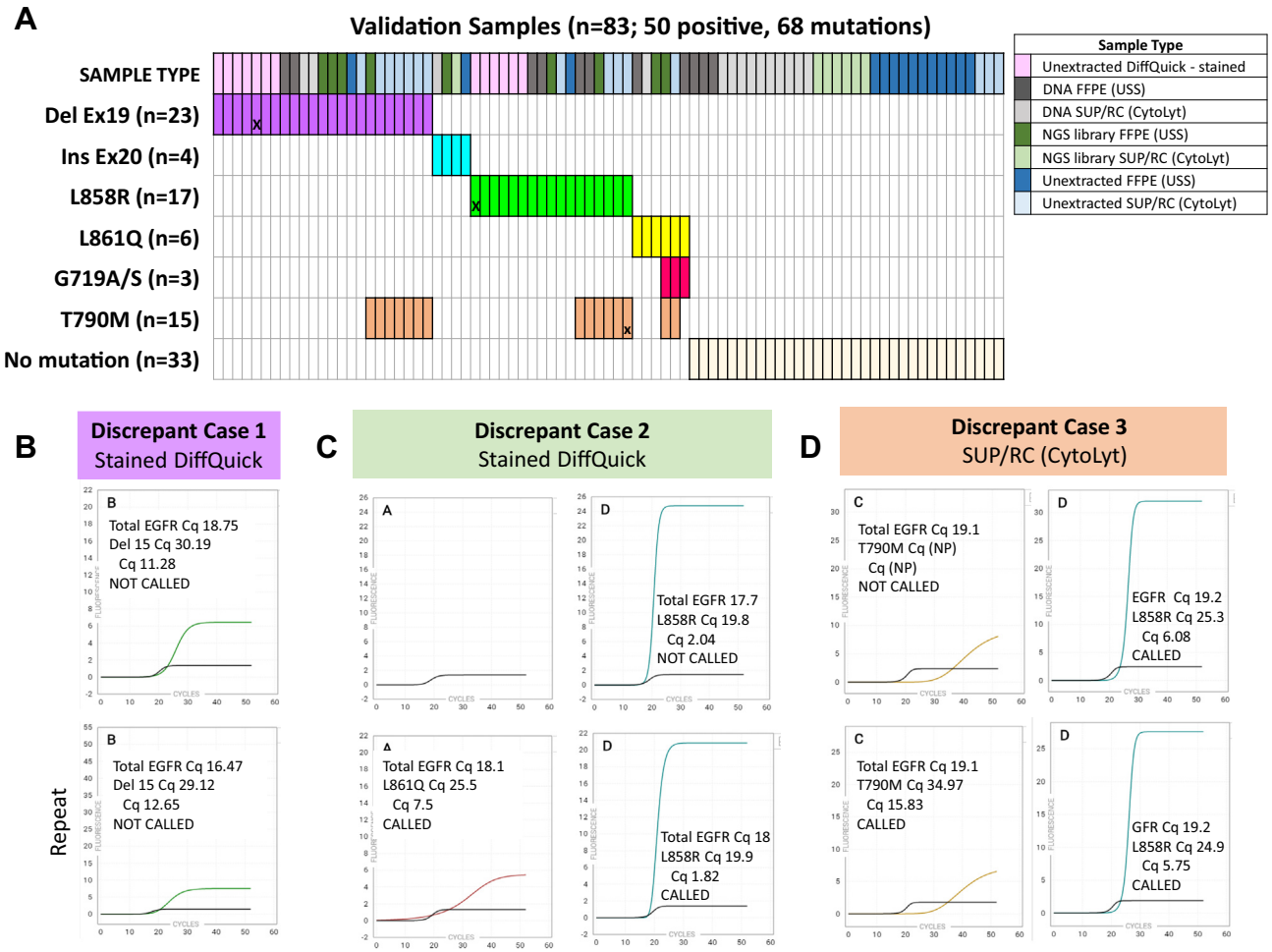

Figure 1. (*A*) Summary of the accuracy study: 83 cases (50 positive, 33 negative) were tested; 16 cases had two or three co-occurring mutations each, totaling 68 mutations. Sample type is denoted in the top row for each case, and discrepancies with the reference method are marked with an X. Three discrepancies were identified using the Idylla automated calls, two associated with Diff-Quick slides (stained). One discrepancy involved a subclonal T790M mutation. (*B*) Discrepant case 1: mutation in track B (Del15, green line) was not called by the automated software. Manual review of PCR tracings reveals the mutation curve with expected sigmoid shape and long plateau but slightly delayed ΔCq of approximately 11.5 and low fluorescence. Repeat testing (bottom) with higher input reveals same pattern without a mutation call. (*C*) Discrepant case 2: L858R mutation not called by the automated caller despite the presence of a valid curve with Cq and ΔCq within expected parameters for the mutation. On repeat testing, the L858R mutation is called but a false-positive L861Q is also generated (track A). Although the L861Q meets the calling criteria on the basis of Cq and ΔCq, the PCR curve reveals a low slope which is not expected when the mutant curve rises at the same time as the total *EGFR* curve. Low slopes are generally found with markedly delayed ΔCqs. (*D*) Discrepant case 3: resistance sample with L858R mutation (VAF 9%) and T790M (VAF 3%). Subclonal T790M not called on initial testing but called on repeat testing. Cq, cycle of quantification; FFPE, formalin-fixed paraffin-embedded; NGS, next-generation sequencing; NP, not provided on the platform output; PCR, polymerase chain reaction; SUP/RC, supernatant or residual cytology; USS, unstained slides; VAF, variant allele frequency; ΔCq, difference between the Cq of mutant *EGFR* and total *EGFR* from the same chamber.

corresponding to a diagnostic sensitivity of 94.0% (95% confidence interval [CI]: 83.5%–98.8%), specificity of 100.0% (95% CI: 89.4%–100.0%), positive predictive value of 100.0%, and negative predictive value of 91.7% (95% CI: 78.6%–99.3%) (Fig. 1*A*). Overall, the diagnostic accuracy of the Idylla assay (i.e., the overall probability that a patient was correctly classified) in this cohort was 96.4% (95% CI: 89.8%–99.3%). Of note, the three *EGFR* mutations that were not correctly called by the Idylla software (two from stained slides, one from a resistance sample with subclonal T790M mutation) had mutation curves on manual review of the unfiltered data. Repeat testing with higher input resolved two of the three but also generated a false-positive call in one stained slide sample (Fig. 1*B*–*D*).

The analytical sensitivity study revealed a variable limit of detection depending on the total DNA input and the specific variant tested (Fig. 2*A*, Supplementary

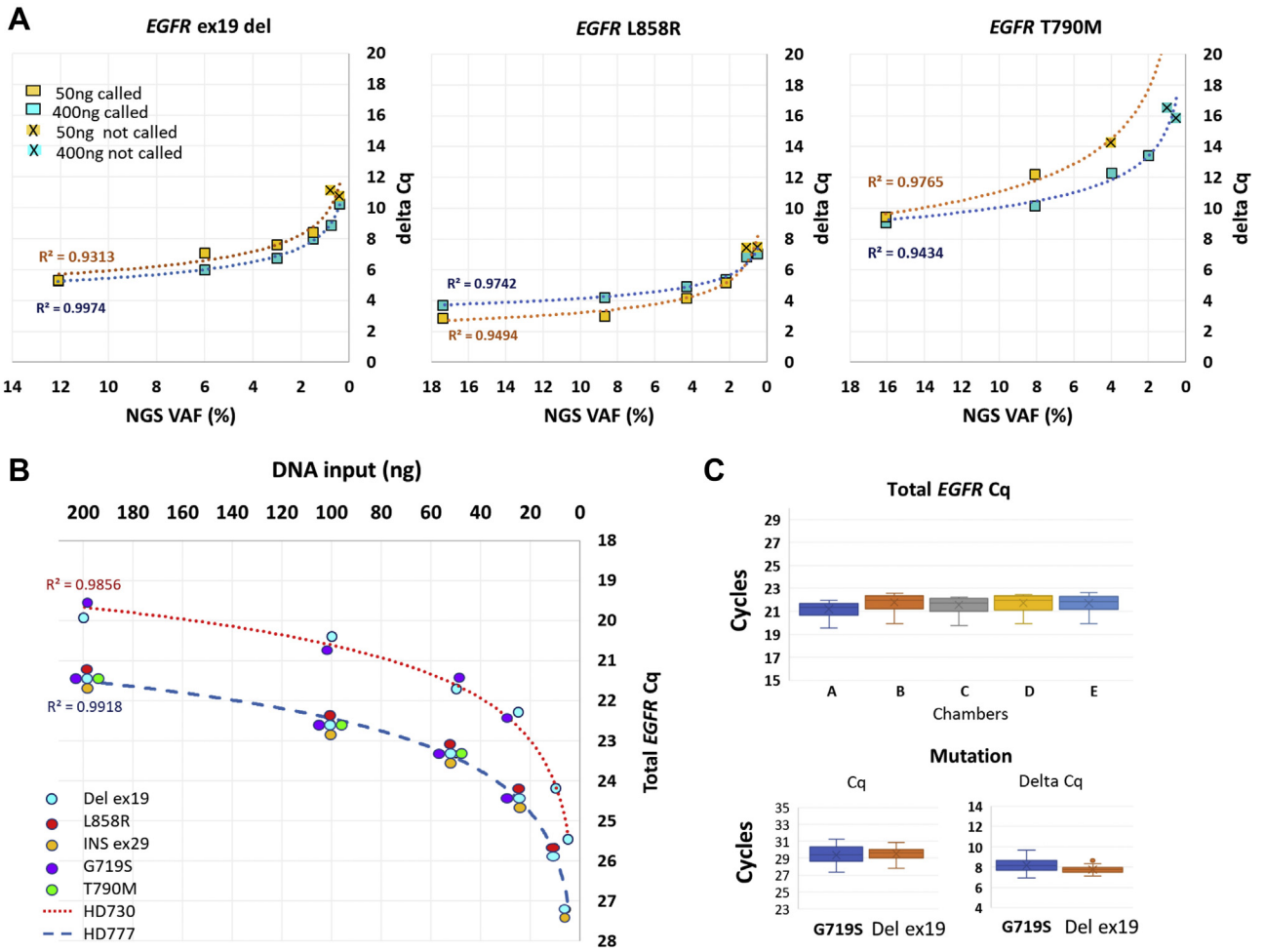

Figure 2. (*A*) Results of the sensitivity study for *EGFR* Idylla assay. Sequential dilution levels of the mixed positive control sample (three mutations) with *EGFR* wild-type DNA are tested with two inputs (50 ng and 400 ng) to assess detection. The corresponding VAFs for each mutation at each dilution point are plotted on the X axis with the resulting ΔCq on the Y axis. At fixed inputs of 50 ng and 400 ng (DNA from non-FFPE sample), the total *EGFR* Cq remains stable, averaging 21.8 and 18.8, respectively. The ΔCqs are inversely proportional to the amount of target nucleic acid in the sample as revealed by the sequential increase in ΔCq as the VAF for each mutation decreases. At each individual dilution point, the ΔCq is similar for the 50 ng and 400 ng inputs for the *EGFR*-sensitizing mutations but drifts apart for the T790M owing to inconsistent detection and lack of plateau. Using the 50 ng input, both sensitizing mutations are detected at VAF approximately of 1% to 2%. At lower VAFs, the signal is detected but does not meet the full criteria for automated mutation calling (not called) but can be flagged as equivocal. The limit of detection for the T790M mutation is comparatively lower between 4% and 8%. Higher detection can be attained with the 400 ng, below 1% for the sensitizing mutations and approximately 2% for T790M. (*B*) Results of the minimum input study. The following two positive controls are used: top (dotted red line) corresponds to control HD730 (del ex19 at 5% and G719S at 14%) and bottom (blue dashed line) corresponds to HD777 with five mutations, all at 5% VAF. Differences in total *EGFR* Cq at each input are because of the lower quality of the HD777 template (highly fragmented DNA) compared with non-FFPE sample material. The total *EGFR* Cqs are inversely proportional to the total input. Inputs that maintain total *EGFR* Cqs at approximately 23 allow detection of all mutations if present at 5% VAF and above. As total *EGFR* Cq increases, detection capability of the assay decreases depending on the mutation, with the lowest detection for T790M and highest for del ex19. (*C*) Interassay reproducibility. Results of the positive control HD730 tested 32 times across eight different instruments reveal excellent repeatability. Total *EGFR* Cq mutation and calculated ΔCq remain within a tight range (SD = 0.6, CV = 0.03). Cq, cycle of quantification; CV, coefficient of variation; FFPE, formalin-fixed paraffin-embedded; NGS, next-generation sequencing; VAF, variant allele frequency; ΔCq, difference between the Cq of mutant *EGFR* and total *EGFR* from the same chamber.

Table 5), revealing higher detection for canonical sensitizing mutations, *EGFR* exon 19 deletion, and L858R. Using a fixed input of 50 ng, the total *EGFR* Cq was maintained at an average of 21.8 (range, 21.2–22.2), allowing for the detection of canonical sensitizing mutations between 1.5% and 2.2% VAF. With a higher input of 400 ng, sensitizing mutations as low as 0.4% VAF could be detected, corresponding to a total *EGFR* Cq

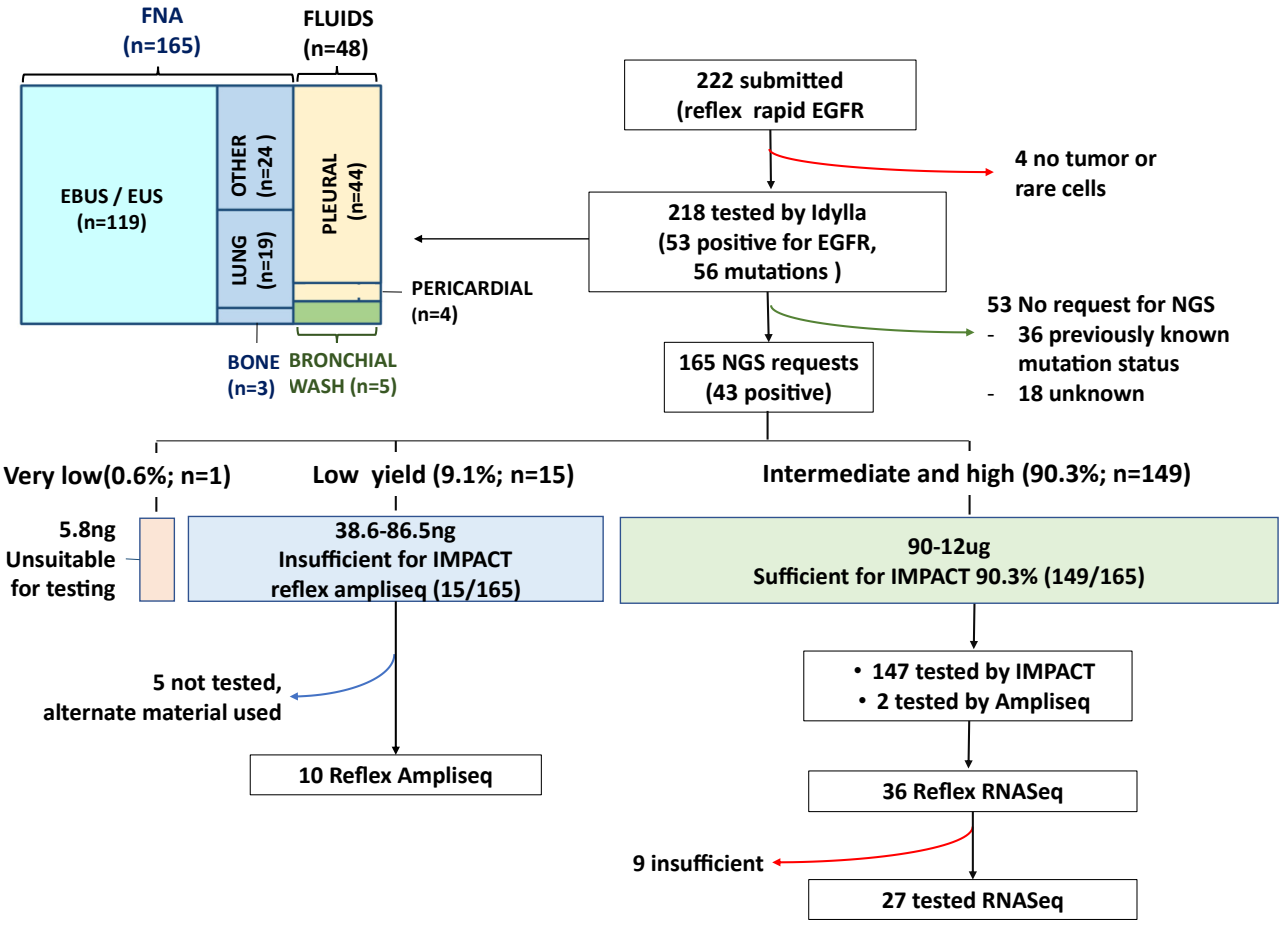

Figure 3. Summary of cases received and tested. Distribution of the sample types summarized on the tree map on the top left corner. EBUS, endobronchial ultrasound; EUS, endoscopic ultrasonography; FNA, fine-needle aspiration; NGS, next-generation sequencing; RNASeq, RNA sequencing.

range of 18.2 to 19.5. The detection of the T790M resistance mutation was comparatively lower, at approximately 8% and 2% VAF for the 50 ng and 400 ng inputs, respectively.

The minimum input study revealed that inputs that maintained the total *EGFR* Cq at less than or equal to 23 allowed for all the mutations to be detected (all present at 5% VAF) (Fig. 2*B*). Sequentially higher total *EGFR* Cqs led to variable loss of mutation detection. The exon 19 deletion and L858R mutation assays had the highest detection capability and could be detected with inputs as low as 10 ng, corresponding to total *EGFR* Cq values as high as 28. The interassay reproducibility study revealed excellent reproducibility, with only minimal differences in Cq and ΔCq values (SD, 0.6; coefficient of variation, 0.03, between runs) (Fig. 2*C*).

On the basis of the validation data, a set of criteria for manual review were established to supplement the automated calling by the Idylla software and allow flagging of equivocal cases or those at risk of false-positive or false-negative results (Supplementary Tables 6 and 7).

Clinical Implementation of the Assay and Comparison With NGS Testing

In total, 222 cytology samples were received for routine molecular testing between September 13, 2018, and November 11, 2019 (Figs. 3 and 4). Four cases had no or only rare tumor cells in the corresponding stained slide and were deemed insufficient for testing. Idylla *EGFR* was performed on 218 samples, including 47 supernatant or residual cytology samples in CytoLyt, 167 FFPE unstained slides, and four extracted DNA from FFPE (≤10 total slides with tissue received). In all, 24.3% (53 of 218) were positive for an *EGFR* mutation by Idylla (56 mutations, Fig. 4). The mean total *EGFR* Cq value was 22.3 (range, 13.6–29.1); 7.3% (16 of 218) were flagged in the high-risk category as potential false negatives owing to high total *EGFR* Cq and relatively low tumor content, and 1.4% (3 of 218) of cases were flagged as equivocal and retested to confirm the mutations (Fig. 5). No test failures were encountered (Fig. 4), and TAT averaged 2 days from receipt of tissue to release of the final report into the electronic medical record.

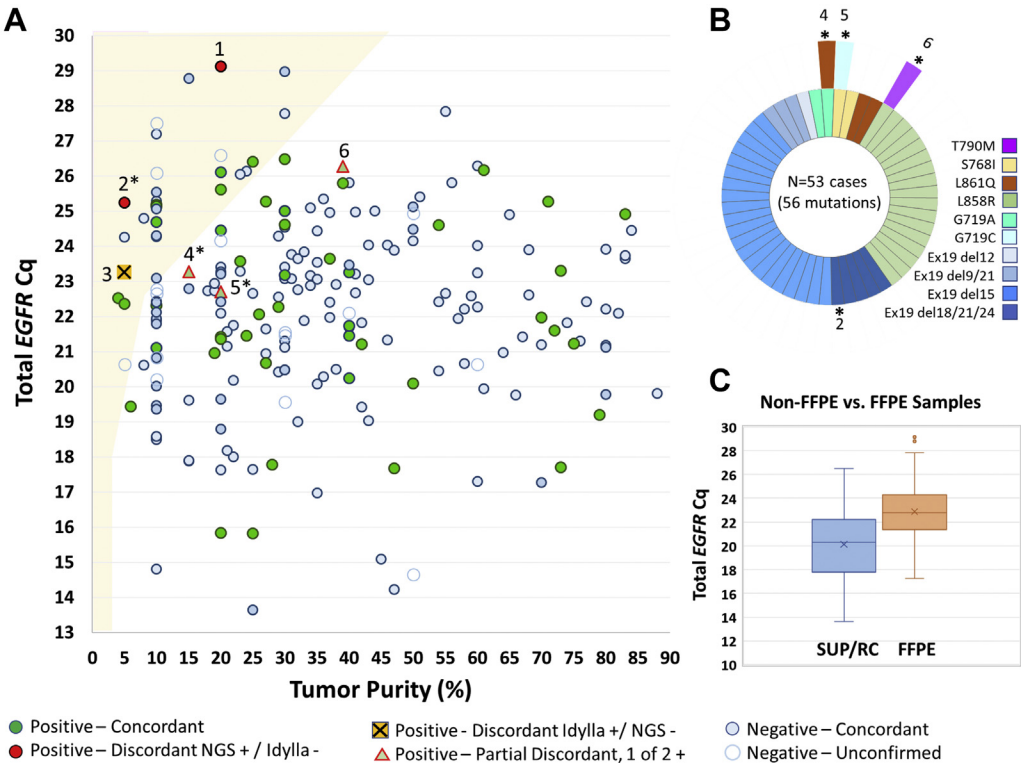

Figure 4. (*A*) Results of 218 cases tested by Idylla, in which plotting is on the basis of tumor content (x axis) and total *EGFR* Cq (y axis). Positive samples are in green, and negative samples in blue, with 159 confirmed on the basis of concurrent NGS testing. Cases with no NGS requests were correlated with existing records of known mutational status from alternate testing. A total of 17 negative cases had no other test performed and were considered unconfirmed. The shaded area denotes the region at risk for false-negative results on the basis of high total *EGFR* Cq (surrogate metric for low input) and relatively low tumor content. Positive results seen inside the boundaries of this region are canonical sensitizing mutations, which have better limit of detection; all negative results in this area should be interpreted with caution. Six discordant cases were identified. Five were not called by the Idylla software (three flagged as equivocal (*) on the basis of manual review and prompted confirmatory testing). Case 3 was not called by NGS but had low-level read support below 1% on manual review. Three partial discrepancies were found near the shaded region (cases 4, 5, and 6). All three cases were double mutant with one of the mutations not called by Idylla. Details of cases 2, 4, and 5 are illustrated in Figure 5. Case 6 had a subclonal T790M mutation at 9% which could not be detected given the elongated Cq of approximately 26, despite the high tumor purity. (*B*) Distribution of the 56 mutations (53 cases) detected by the Idylla system among the 218 cases tested. (*C*) Comparison of total *EGFR* Cq of samples after stratification on the basis of fixation. Despite very minimal material utilized, the SUP/RC revealed significantly lower total *EGFR* Cqs than FFPE sections, with median total *EGFR* Cq of 20.29 versus 22.77, respectively ($p < 0.00001$). The total *EGFR* Cq is a surrogate measure of effective nucleic acid template. Lower values are associated with higher sensitivity. Cases with total *EGFR* Cq of 20 or less allow detection of mutations below 2% compared with greater than 5% for total Cq levels above 23. Cq, cycle of quantification; FFPE, formalin-fixed paraffin-embedded; NGS, next-generation sequencing; SUP/RC, supernatant or residual cytology.

NGS was requested for 75.7% (165 of 218) of the cases and performed on 96.4% (159 of 165), 89.1% (147 of 165) by MSK-IMPACT, and 7.3% (12 of 165) by MSK-AmpliSeq on the basis of DNA yield and specific request (Fig. 3) with median coverages of 614× and 1038× for the two assays, respectively. The TAT for comprehensive NGS testing ranged from 2 weeks to 4 weeks. Among the samples tested by NGS, 32.7% (52 of 159) were positive for *EGFR* mutations (60 mutations) with 17 additional mutations (nine patients) not previously detected by Idylla: two owing to insufficient sensitivity associated with long total *EGFR* Cq (cases 1 and 6 in Fig. 4), and 15 not included in the Idylla assay design (Fig. 6 and Supplementary Table 8). The overall concordance of Idylla with NGS (for those mutations within the Idylla assay design) was 96.2% (153 of 159) when using automated calling only and 98.1% (156 of 159) with incorporation of manual review and confirmation of three equivocal cases. One mutation called by Idylla was not called by NGS (case 3); manual review of the NGS data revealed low-level read support (<1%), which was below the established threshold for mutation calling. The diagnostic sensitivity for the Idylla assay with manual review was therefore 95.6% (95% CI: 84.9%–99.46%), with 100% specificity.

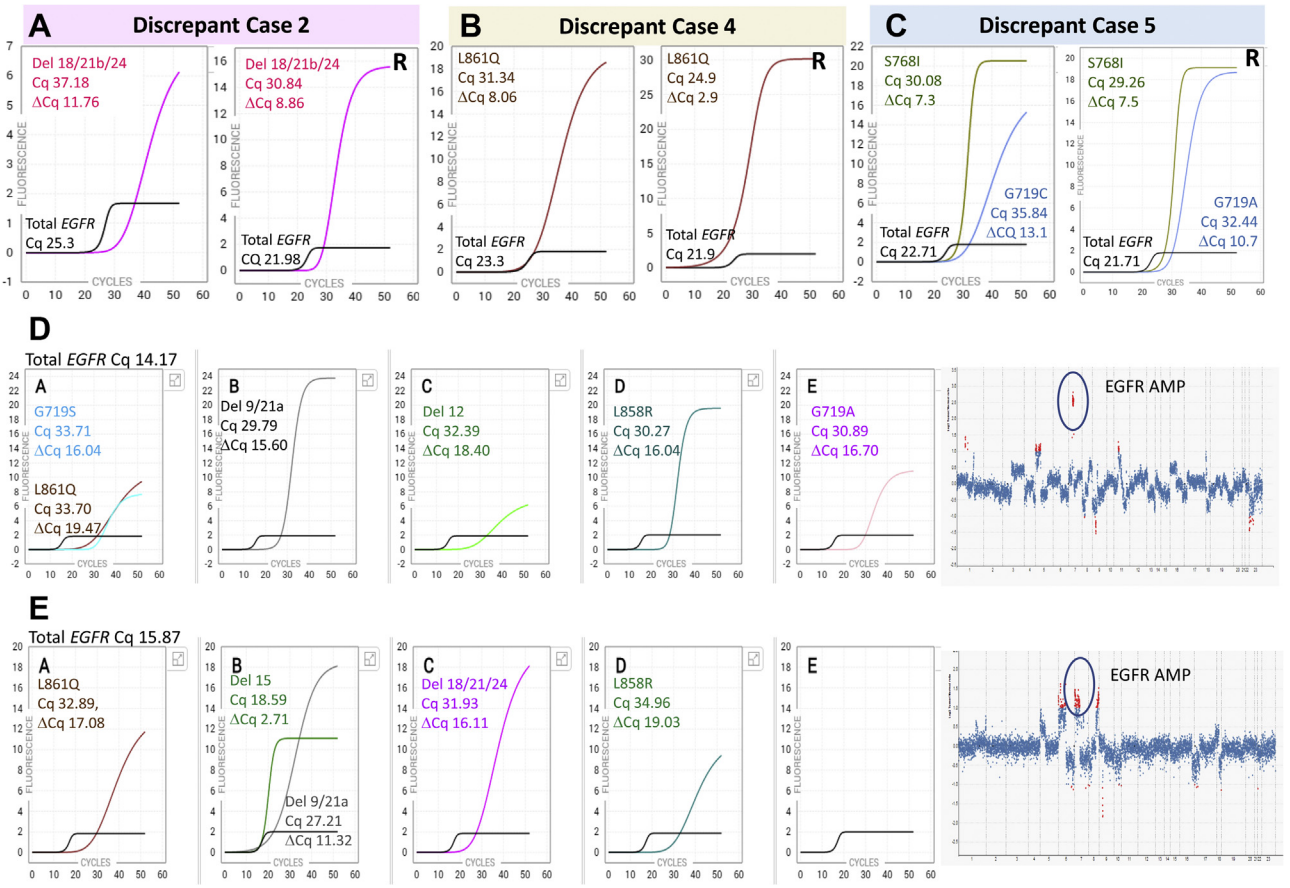

Figure 5. Discrepancies and example cases: discrepant cases at the top reveal the initial result on the left and repeat testing on the right, demarcated "R." (*A*) Discrepant case 2 is an example of low input (underloaded cartridge) on the basis of the total *EGFR* Cq of 25.3. The estimated tumor content was approximately 5%—in this context a mutation, if present, would be expected at approximately 2% to 3% variant frequency (assuming no associated amplification). Detection of a mutation at this level would require a total *EGFR* Cq between 18 and 19. The case was flagged as equivocal on the basis of the presence of valid mutation curve with a steep slope and delayed Cq (as expected for the tumor content) but still within the calling range. On the basis of these characteristics, it is likely to be mutated rather than an artifact. Repeat testing with higher input confirms the mutation. (*B*) Discrepant case 4 is a normal input sample (normal loaded cartridge) on the basis of a total *EGFR* Cq of 23 and estimated tumor content of 15%. This is a double mutant case with both G719A and L861Q mutations; however, L861Q is not called owing to a delayed plateau. This case was flagged as equivocal on the basis of the high intensity of the signal, with curve rising at the same time as total *EGFR* with steep slope and ΔCq within the expected range. Repeat testing with higher input confirms the mutation. (*C*) Discrepant case 5—normal input sample with two mutations with G719C not initially called by the system owing to lack of plateau but a ΔCq within expected range for the mutation. The case was flagged as equivocal, and repeat testing reveals both mutations with high signal and similar plateau. (*D*) Example of an overloaded cartridge. Cases with total *EGFR* Cq between 13 and 17 are considered overloaded owing to high input or very high amplification. In the setting of very high amplification, there is a risk for high noise and mispriming, which is recognized as multiple valid curves that meet several of the shape and slope criteria for mutation calls. Repeat testing with lower input is advised in these cases to lower the total *EGFR* Cq. Higher input increases the noise level and leads to false-positive calls. Concurrent NGS testing in this case reveals high *EGFR* amplification as seen in the copy number plot on the right, but no *EGFR* mutation was detected. (*E*) Example of an overloaded cartridge with an *EGFR* mutation and high amplification. In cases with an *EGFR* mutation and concurrent high amplification, an overloaded pattern is also found and may lead to more than one mutation being called. In these cases, the true mutation is recognized by the low ΔCq. In this case, the mutations are deletion 15; AMP, amplification; Cq, cycle of quantification; ΔCq, Delta Cq - difference between the Cq of mutant *EGFR* and total *EGFR* from the same chamber; PCR, polymerase chain reaction.

Among the cases tested by NGS, 101 clinically actionable alterations of levels 1 to 3b, on the basis of OncoKB[37] criteria, were detected in 60.4% (96 of 159) of the cases. The genomic landscape of the most common driver alterations and other mutations is depicted in Figure 6. A total of 36 cases were reflexed for additional testing on RNA using the MSK-Fusion solid assay, 75.0% (27 of 36) of which were successfully tested to detect or confirm eight clinically relevant structural variants: four *MET* exon 14 skipping mutations, two *ALK* fusions, one *EGFR* kinase domain duplication, and one *NTRK1* rearrangement. Overall, 2.5% (4 of 159) of the cases had no

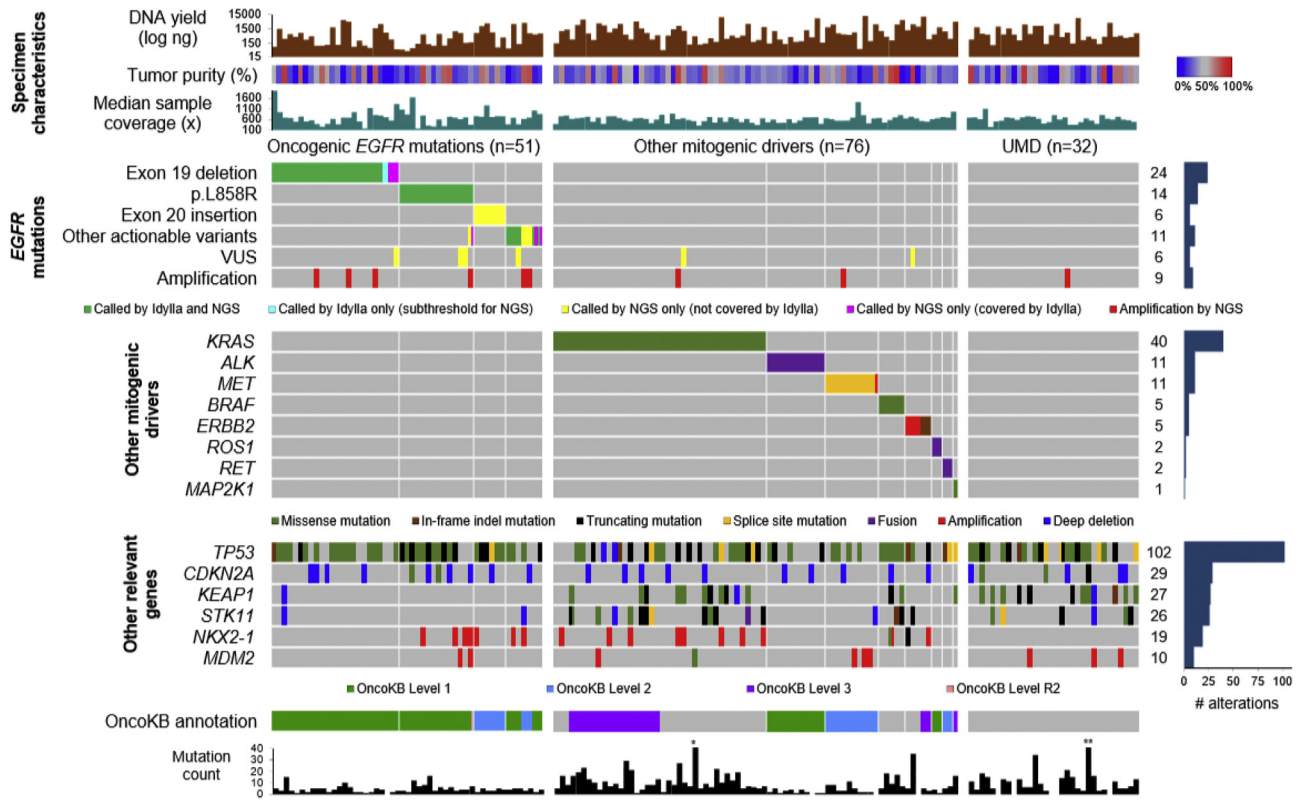

Figure 6. Summary of the specimen characteristics and genomic findings from Idylla and NGS. The samples are divided into the following three groups: those with oncogenic *EGFR* mutations (left), those with other mitogenic drivers (middle), and those with unknown mitogenic drivers (right). The specimen characteristics include total DNA yield, tumor purity, and median sample coverage from NGS. For cases with oncogenic *EGFR* mutations, the distribution of specific *EGFR* variants is provided. For cases without oncogenic *EGFR* mutations, the distribution of other mitogenic driver alterations is provided. For all cases, the mutational status of other relevant genes in lung cancer is provided at the bottom. In 96 cases (60%), actionable genetic alterations (i.e., variants with OncoKB levels 1–3 and R1–R2 annotations) were detected. The bottom panel reveals the total count of sequence mutations (i.e., mutational count) for each sample. Two samples had mutational counts greater than 40: one case with 48 mutations (*) and another with 84 (**). NGS, next-generation sequencing; UMD, unknown mitogenic drivers; VUS, variants of unknown significance.

detectable somatic alterations in the context of low tumor content (<10%), raising the possibility of false-negative results, and 20.8% (33 of 159) had tumor content of 20% or less, potentially limiting the assessment of copy number alterations and fusions. Somatic alterations ranged from one to 86 among the 155 cases with detectable events.

Discussion

Institutions providing care for patients with NSCLC are increasingly adopting NGS for comprehensive molecular profiling of tumor samples. However, there are limitations to utilizing NGS as a single test. Therefore, an integrated multitest approach remains essential for both timely and comprehensive assessment. Given the high incidence of *EGFR* mutations in lung adenocarcinoma, ranging from 15% to 40% depending on the population, prioritizing assessment of this marker by a rapid assay would represent a critical step in guiding initial treatment decisions. The success rate for small biopsies and cytology samples with this multitest approach is determined by the appropriate choice of technology and optimization of protocols to maximize tissue use and maintain high performance across all assays.

Our group has previously reported that process optimization in tissue handling and extraction maximizes nucleic acid yield for successful upfront NGS testing in approximately 90% of all cytology cases.[21,27,38] Specifically looking at endobronchial ultrasonography-transbronchial needle aspiration samples, a recent study from our institution also revealed a marked improvement, from 76.3% to 92.3%, across a 2-year period (2014–2016) of process optimization.[27] In this study, we further report that the incorporation of rapid *EGFR* assessment using the Idylla platform does not affect, but rather improves, the success rate of subsequent comprehensive NGS profiling, while allowing rapid stratification of patients for treatment with *EGFR* targeted therapy (2 d versus 2–4 wk) and those in need for more comprehensive testing.

The Idylla system is a platform specifically marketed for use on unextracted material directly from FFPE sections but, with proper validation, is also adaptable to other tissue sources in the clinical setting. The cartridge-based design allows testing of single samples on demand without the need for batching, with minimal hands-on time (2 min) and delivering results in less than 2 hours. The platform can be easily integrated into any clinical molecular diagnostic laboratory, enabling rapid triaging for critical molecular markers. Although several publications have already documented the use of Idylla for FFPE biopsy samples,[39–45] descriptions of the suitability of cytology samples for this platform have remained confined to a few small, proof-of-principle studies,[46] and the corresponding performance characteristics, clinical validation, and extended clinical experience have remained largely undefined. The application to cytologic material is ideal, however, as it can easily address the issues of TAT and also provide rapid assessment in samples that are very scant and otherwise unsuitable for any other kind of testing.

Through our extensive validation of the Idylla system for *EGFR* assessment, we reported that any cytology sample may be suitable for testing and the assay performs robustly for initial screening and detection of canonical sensitizing mutations within the design of the assay. By contrast, the detection of the T790M mutation is relatively lower which, together with the absence of primers for other mutations, such as C797S and G724S, makes this assay unsuitable for the assessment of patients in the setting of resistance. A notable benefit for small samples is that the test itself is based on a tissue lysate that is internally prepared in the cartridge, thereby, avoiding large tissue losses through the multiple vial transfers and clean-up steps associated with standard DNA extraction protocols. Using unextracted tissue, however, has important adverse implications on the preanalytic assessment of tissue suitability and quantification, with direct impact on assay sensitivity. Metrics, such as DNA quantity and quality, for example, cannot be used as a guide for template input. Furthermore, because the density of the cells can vary widely depending on the sample type and preparation, estimating the precise input load can be challenging. Both overloading and underloading may adversely impact test performance and interpretation; therefore, the user must be familiar with the system and expected parameters to obtain accurate results.

Given the variability of tissue quality and quantity of unextracted material, general guides for tissue loading are best defined and established by each laboratory based on their own samples. In our case, on the basis of our validation data of non-FFPE specimens, approximately 3000 cell equivalents (20–25 ng of DNA) were needed to consistently maintain detection of all the variants at 5% VAF, whereas for FFPE samples, twice the input was required. In practice, we generally use three FFPE sections (maximum of five, at 5 μm thickness) and aim for greater than or equal to 10% tumor. We note, however, that analytical sensitivity varies among the individual assays in the cartridge, such that some alterations (exon 19 deletions and L858R mutations) could be detected at VAFs well below 5% even with inputs as low as 5 ng. Still, the definitive assessment of tissue suitability can only be made after testing, on the basis of the total *EGFR* Cq of the sample, in combination with the estimated tumor content. In this regard, the supernatants and minimal residual cytologic material far outperform the FFPE tissue, generally yielding lower total *EGFR* Cqs and, therefore, providing higher sensitivity.

As a general guide, total *EGFR* Cqs of less than or equal to 23 must be maintained to ensure detection of all mutations at 5% VAF. More sensitive detection can be attained with lower values, approaching 1% VAF when total *EGFR* Cq is approximately 18 or lower. High tissue input is needed to reach this limit of detection which is not possible in very scant samples, particularly FFPE preparations. For scant samples that cannot be split for both NGS and *EGFR* Idylla, precapture NGS libraries can be prepared, and an aliquot can be used for Idylla to enable dual testing. The libraries are also a valuable source of material for repeats, confirmations, or other assays, as they allow more accurate quantification of DNA for loading. In addition, although we generally aim for at least 10% tumor, we often test samples with lower tumor proportion given that *EGFR* mutations, if present, are often associated with concurrent *EGFR* amplification of the mutated allele, enabling their detection at higher VAFs than expected for the tumor content. If the sample is negative, however, a false-negative result cannot be ruled out, and adequate disclaimers must be reported.

Among the cytology samples validated in this study, Diff-Quick stained smears deserve special mention as the stain could adversely affect automated mutation calling owing to high background noise and interference with the fluorescent signal. This has also been reported in a pilot study by De Luca et al.,[46] in which the authors reported false negatives in approximately 20% of cases but no false positives. In our validation study, we noted that the stain could lead to both false-negative and false-positive calls using automated calling. Manual review of the unfiltered quantitative PCR (qPCR) tracings allows for flagging of these cases as equivocal, but orthogonal confirmation is required. For this reason, if a stained aspirate smear is the only sample available for testing, we recommend destaining before performing the assay, which may improve the success rate, but further

validation with side-by-side comparison is still required for this type of sample.

In our extended experience with the Idylla platform across more than 1200 unstained NSCLC cases, including small biopsies and cytology samples, we have not encountered any false-positive calls generated by the automated Idylla software. Conversely, assay failures are exceedingly rare, even in the context of minimal tissue loading. Therefore, samples with low input and low tumor content are most concerning and at high risk of false-negative results, highlighting the importance of identifying these cases for proper reporting as insufficient rather than negative (Supplementary Table 6). On the basis of our experience, we emphasize that no diagnosis should be made solely using the automated calls of the system. Rather, manual review of all qPCR tracings should be incorporated as part of routine clinical assessment for proper analysis and flagging of cases requiring confirmatory testing. We specifically note a propensity for false-negative calls involving codons G719, L861, and S768 even with adequate tumor content, owing to lack of plateauing of the qPCR curves (Fig. 4). We also note the propensity for low-level mispriming in cases with high *EGFR* amplification and cartridge overload (Fig. 5*D* and *E*), which could potentially lead to false-positive calls or cause confusion during manual review and interpretation. To resolve these issues in a scalable manner, the raw data may be also analyzed using in-house–developed algorithms, which in our case have proven helpful for fine tuning of mutation calling criteria and automated flagging of cases needing further review.

In our 1-year experience following the incorporation of rapid *EGFR* testing by Idylla, the success rates for NGS testing have remained above 90% with results that are in keeping with our previous reports.[47] Of the 165 cases with NGS requests, 99% had sufficient DNA yield for testing (91% for MSK-IMPACT and 8% for the smaller MSK-AmpliSeq panel). This was, in large part, owing to the repurposing of supernatants or residual scant cytologic material from ThinPrep samples (previously discarded) as the primary source of tissue for expedited Idylla testing and preserving the cell block as a backup for NGS. An additional step in repurposing the material was the use of residual lysed cell material (lysate) from our automated DNA extraction process for RNA extraction. This material allowed us to successfully test 75% of all cases reflexed to the MSK-Fusion solid panel, enabling the detection and confirmation of several actionable gene rearrangements involving *ALK*, *EGFR*, *MET*, and *NTRK1*. With further adjustments to our workflow, the success rate for targeted RNA sequencing could be further improved. Although most cytology samples had ample material for testing, approximately 12% of cases had tumor content at or below 10%, which could affect the detection of the full spectrum of genetic alterations, particularly copy number alterations, tumor mutation burden, and microsatellite instability status.

At present, given that the diagnosis of lung cancer requires morphologic and immunophenotypic confirmation, initial tumor profiling should be performed on the diagnostic tissue for accurate and specific assessment. Although cytologic samples, by virtue of their limited tissue content, have been a source of concern, in this study, we found that most samples that are suitable for a cancer diagnosis by morphology are also suitable for comprehensive genomic profiling. A multitest approach that prioritizes the most common and immediately actionable alterations is feasible and does not compromise further comprehensive testing provided that all steps of tissue acquisition and handling are well optimized. We emphasize the need for a cohesive workflow among the clinical, cytopathology, molecular, and bioinformatics teams as a pivotal component in the success rate of molecular testing.

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Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *Journal of Thoracic Oncology Clinical and Research Reports* at www.jtocrr.org and at https://doi.org/10.1016/j.jtocrr.2020.100077.

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