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End-to-end automation for the in-house detection of targeted cancer mutations in *KRAS*, *NRAS*, *BRAF* and *EGFR*

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Keywords

Nonstandard Abbreviations

KRAS, Kirsten rat sarcoma viral oncogene homolog gene; *NRAS*, neuroblastoma RAS viral oncogene homolog gene; *BRAF*, B-Raf proto-oncogene; *EGFR*, epidermal growth factor receptor gene; FFPE, formalin-fixed paraffin-embedded; LOD, limit of detection; TAT, turnaround time; NSCLC, non-small cell lung cancer; mCRC, metastatic colorectal cancer; NGS, Next-generation sequencing; PCR, polymerase chain reaction; Cq, cycle of quantification; VAF, variant allele frequency; NCCN, National Comprehensive Cancer Network.

List of human genes discussed

KRAS, *NRAS*, *BRAF*, *EGFR*

Keywords

cancer mutation testing, *KRAS*, *NRAS*, *BRAF*, *EGFR*, Idylla, test automation, local testing, diagnostic performance

Abstract

Background: The Idylla mutation test is a fully automated, real-time PCR-based assay for the targeted detection of cancer mutations in the key oncogenes *KRAS*, *NRAS*, *BRAF* and *EGFR*. The assay has a quick turnaround time and is cost effective lending itself for local implementation in a hospital setting.

Methods: The Idylla mutation test was validated on a total of 182 formalin-fixed paraffin-embedded (FFPE) cancer tissue samples, and a retrospective review of 115 patients who had the Idylla mutation test ordered at Tufts Medical Center (Boston, USA) was performed over 18 months.

Results: The validation yielded 100% sensitivity, 100% specificity, 100% overall accuracy, positive predictive value of 100%, and negative predictive value of 100%. The limit of detection (LOD) was ≤1%. The average turnaround time (TAT) from surgical specimen retrieval to result reporting was 14.7 days, from specimen receipt in the testing laboratory to reporting 2.8 days. The mutation detection rate was 27.4%.

Conclusions: The Idylla mutation test is highly accurate. To obtain the possible short turnaround time of the assay, efficient intradepartmental workflows are critical. The use of the assay in non-small cell lung cancer (NSCLC) is beneficial to resections in tumor stages 1-3. In cases where the Idylla groups multiple mutations into one reporting category, the actual underlying mutation cannot be identified. In instances where ambiguous results have clinical implications, as well as for normal Idylla results, follow-up with Next Generation Sequencing (NGS) is warranted.

Impact Statement

With standards in molecular diagnostics evolving, we reassess the Idylla mutation test based on our validation and 18-month experience. We demonstrate the potential for reliable positive results, avoiding the need for expensive and time consuming NGS analyses in many cases. We address workflow challenges to achieve short turnaround times, and other limitations. This work makes an important contribution to help understand better the strengths, challenges, and weaknesses of the Idylla when implementing this increasingly popular, fully automated platform for cancer diagnosis in a hospital setting.

1. Introduction

Molecular testing for actionable mutations in known oncogenes plays an increasing role in the disease classification, treatment, and enrollment in clinical trials (1-4), especially in non-small-cell lung cancer (NSCLC), in metastatic colorectal cancer (mCRC), and cutaneous melanoma. Much of the genetic testing is now performed in commercial laboratories that are geared toward high-tech, high throughput Next Generation Sequencing (NGS). Although the cost for NGS has fallen dramatically in the past decade, establishing the latest high throughput platforms is prohibitive in most tertiary care settings, usually due to limited throughput yet immense capital and operational costs. Despite the technical advancements of NGS technologies, the turnaround time for high-throughput commercial laboratories takes 2 to 4 weeks. Tertiary care centers are seeking for ways to perform in-house testing with short turnaround time (5, 6). In recent years the Idylla platform has gained popularity. Here we describe our validation and 18-month experience with the Idylla mutation test at Tufts Medical Center (Boston, MA) and detail some of the lessons learned.

2. Materials and Methods

2.1. Instrumentation and assay design

The Idylla (Biocartis, Mechelen, Belgium) is a cartridge-based platform that provides fully automated processing from sample loading to cloud-based result reporting (7, 8). Per platform one sample can be processed at any given time. Currently, four unique gene-specific test

cartridges are available that target actionable mutations in the oncogenes *KRAS, NRAS, BRAF*, and *EGFR* (Supplemental Tables 1-4).

Per manufacturer's specifications, the Idylla assay requires a neoplastic cell content of ≥50% per specimen for testing with the BRAF cartridge, and ≥10% for *KRAS*, *EGFR*, *NRAS*/*BRAF* testing (on a combined *NRAS-BRAF* cartridge, Supplemental Table 5). To avoid contamination, the specimen is carefully placed with a sterile pincer between 2 small Whatman filter papers (10 mm) wetted with nuclease-free water. Subsequently, the specimen is inserted into the Idylla cartridge and loaded on the instrument. The shortest run time is noted with the *BRAF* cartridge (90 minutes), while the assay takes longest with the *EGFR* cartridge (150 minutes). The Idylla mutation assay is a real-time multiplex PCR based on PlexPrime™ and PlexZyme™ (also known as MNAzyme) chemistry (9). All reagents and controls are included in the cartridge. The assay runs fully automated through deparaffinization, tissue digestion, DNA liberation, and allele-specific target amplification. During these reactions, the amplicons are labeled with fluorescent dyes to allow for the capture of fluorescent target signals during several PCR cycles. The total DNA input in each chamber serves as the internal processing control that is analyzed together with the actual mutation targets. Data analysis is performed with software proprietary to Biocartis. The fluorescent signals are then translated into qualitative calls for the reaction-specific PCR curves. Of specific interest are the cycle of quantification (Cq) values of the amplicons for the individual mutations, the control signals that measure the total DNA input (total Cq), and the difference (ΔCq) between these two parameters. In each chamber, the amplification curve of the target is compared to the expected amplification curve based on DNA input.

2.2. Clinical samples and study design

From July to September 2020, the Idylla mutation test was validated in the CAP/CLIA accredited genetics laboratory at Tufts Medical Center to demonstrate acceptable test accuracy, reproducibility, and the limit of detection. After test launch, the performance of the test was assessed retrospectively for the time frame October 2020 to March 2022. This study was carried out to establish in-house molecular testing for cancer patients at the author's institution. With this scope, the study did not require formal IRB approval or patient consent. The authors worked with anonymized data and did not link these data to any other sources of identifiable data.

Formalin-fixed paraffin-embedded (FFPE) biopsy or resection specimens from patients with non-small cell lung cancer (NSCLC) at the author's institution were retrieved from the biorepository (primary cancer or metastatic tissue). A curl and H&E stained slide from the same block were prepared in the histology laboratory of the department. Macro-dissections were not available. The slide was assessed by a pathologist for specimen adequacy. The validation pool of mutations was supplemented with reference standards from Horizon Discovery, Ltd, Lafayette, CO. A total of 204 samples were tested on the Idylla platform with cartridges for *KRAS*, *EGFR*, *BRAF* and *NRAS* mutations. The Idylla results were compared against the clinical test reports previously obtained (2016-2019) from a CAP/CLIA accredited, commercial reference laboratory performing Next Generation Sequencing with the Illumina MiSeq (San Diego, CA) on specimens from the same tissue blocks.

2.3. Statistical analysis

We computed the sample size for the validation by setting the threshold for sensitivity and specificity of the validation at 90% or higher. Our unit of observation was the individual sample. We aimed to have the precision of about 5 percentage points on each side of 90% to have the lower and upper bound of the 95% confidence interval to be 85% to 95%. With the sample size of 160, the width of the confidence interval is 10%, and the boundary is between 84% and 94% (Supplemental Table 6). We aimed for at least 160 samples in the validation. For the statistical analysis, we computed sensitivity, specificity and their respective 95% confidence intervals to report on overall accuracy, positive predictive value, and negative predictive value (10, 11).

To determine reproducibility, five operators performed the sample set up on the Idylla platform. Ten samples were run twice on different days by different operators. One sample was run twice, on two different days, by the least experienced staff.

According to the manufacturer, the lower limit of detection for the Idylla assay is 5%. Twenty samples from Horizon Discovery had a known variant allele frequency (VAF) ranging from 50% to less than 1% and were used to determine the limit of detection (LOD). Several variants had a VAF of 1%. Seven of the *EGFR* variants and 4 of the *BRAF* variants had VAF≤1%. The lowest allele frequency was known for the c.2369C>T and c.2369C>T variants in *EGFR* (samples HD200 and HD850) with VAF 0.9% (Supplemental Table 7).

According to the manufacturer's instructions the assay should be run within 8 hours after opening a cartridge pouch and entering the sample into the cartridge. To be able to evaluate potential decline of sample integrity after loading the cartridge, we ran 18 samples at different

time intervals after loading the cartridge. Ranging from 5 minutes to 15.5 hours. Results were evaluated for data quality and accuracy.

3. Results

3.1. Validation results

Of the 36 different test results that the Idylla mutation test reports we were able to obtain a range of samples that covered 26 (72%). For the mutation panel on the *KRAS* cartridge 12/21 variants were included in this validation, on the *EGFR* cartridge 10/51, on the *BRAF* cartridge 3/8, and on the *NRAS/BRAF* cartridge 4/18 *NRAF* and 3/5 *BRAF* variants. This included most of the highly prevalent mutations on the gene panels seen previously in the patient population at TMC (Supplemental Tables 1-4). Several samples had to be omitted from the analysis due to missing data (Supplemental Table 8). A total of 182 samples was considered for analysis (Supplemental Table 9). The validation of the Idylla mutation test obtained 100% overall accuracy, 100% sensitivity, 100% specificity, a PPV of 100%, and a NPV of 100% for each cartridge type (Supplemental Table 10). Inter- and intra-operator reproducibility was 100% (Supplemental Table 11). Twenty samples were used to determine the threshold for variant detection. Eleven variants in these samples had an allele frequency of ≤1%. The different time windows from loading the curl on the cartridge to running the assay did not affect result quality. All mutations were identified correctly and data quality for all samples was excellent (Supplemental Table 12). For practical considerations, it is therefore possible to prepare cartridges with a specimen at least 8 hours before the actual start of the test without adverse effect on the results, for example the night before the actual test is run.

3.2. Most frequent test indications and mutation spectrum

From October 2020 to March 2022, we used 135 Idylla cartridges on 115 patients for the detection of cancer mutations (Table 1) in the clinical laboratory. The age range of patients was 6 months to 93 years. The majority of these patients (69.6%) was diagnosed with non-small-cell lung cancer (NSCLC). The Idylla test most frequently ordered was for *EGFR* mutations (65.9%). The second most frequent diagnosis was colorectal cancer (12.7%). For these patients, *KRAS* mutation testing was most frequent (14.1%). Overall, *BRAF* testing was the second most common test performed (22.2%), predominantly for colorectal cancer but also for melanoma. Only a minority of Idylla testing was for melanoma patients (5.2%) because the diagnostic workup for melanoma patients was performed in a reference laboratory. Testing for *NRAS* mutations was very infrequent (8.2%). In the 135 tests performed during the first 18 months after test launch, we identified 37 mutations. This accounts for a mutation detection rate of 27.4% (37/135). For *EGFR* testing on NSCLC specimens, 72 tests were done on adenocarcinomas, and 14 tests on squamous cell carcinomas. Among the tests on adenocarcinomas, 19 tests were mutation positive (14%). All results for squamous cell carcinomas were *EGFR* mutation negative. The most frequently observed mutations in *EGFR* were exon 19 deletions, followed by the L858R mutation. This distribution is concordant with the mutation prevalence previously reported (12, 13). The *KRAS* mutation test was ordered exclusively on colorectal cancer specimens. The most abundant *KRAS* mutation in our patient population was G12D, as seen in other studies (14, 15). With *BRAF* testing, we identified the V600E mutation most frequently. For the collection and analysis of these data, all samples were de-identified to ensure HIPPA compliance.

3.3. Specimen availability and test approach

Running the Idylla on FFPE tissue requires a curl of 5-10 microns. We experienced tissue shortage mostly with lung cancer biopsies. Therefore, we do not use the Idylla mutation test on these specimens but perform NGS which requires significantly less material. For advanced lung cancer stage 4, Her2 mutation testing is part of the routine work up (16). Since Her2 is not included in the Idylla mutation test, a reference lab comprehensively performs all testing for these cases. In colorectal cancer, we perform the Idylla mutation test at any disease stage both on small biopsies and resections. If the *KRAS* mutation status is negative, we reflex to *BRAF* mutation testing (Table 2). In the past 18 months, we encountered several cases in which rapid *BRAF* testing was critical for patients with advanced cutaneous melanoma. Identifying a *BRAF* V600 mutations allows for accelerated therapy decisions. If no immediate decisions are to be made, we opt for NGS testing in melanomas to identify the actual underlying mutation.

3.4. Turnaround time

After the initial 3-month phase that required process improvements, the average turnaround time from receiving the specimen in the test laboratory to the time of result reporting to the oncologist was 2.8 calendar days. Since the Idylla mutation test is ordered as a reflex test after determining the diagnosis (Figures 1 and 2), the overall time from the surgical procedure to the actual result release to the oncologist averaged 14.7 calendar days. During the first 18 months after test launch, we identified additional mutations with the Idylla that were not included in the original validation study. These mutations needed confirmation by an orthogonal method by an off-site

reference laboratory before providing the Idylla test results to the health care provider. This process added 2-4 weeks to the turnaround time.

3.5. Challenges with result interpretation

It has been described previously that the accuracy of mutation detection with the Idylla varies depending on the underlying mutation, with the highest sensitivity for the *EGFR* 15-bp deletion in exon 19 (17). In contrast, the T790M mutation cannot be detected reliably which was attributed to the subclonal nature of the mutation, the uneven distribution of the mutation across tumor tissue, and potential interferences of primer binding in the presence of other mutations (18). The T790M mutation, however, is a clinically relevant mutation in the context of treatment resistance. In our validation study, we identified the *EGFR* T790M mutation correctly.

For a variety of DNA changes (Supplemental Tables 1-4) the Idylla mutation test aggregates different mutations into one reporting category. For example, a change to P, T, or V in codon 146 of exon 4 of the *KRAS* gene is reported as A146P/T/V. The actual underlying DNA change cannot be inferred from the reported result or even the raw data.

When several independent mutations are present in the same gene, the console report indicates only the general detection of a mutation. The actual changes then need to be retrieved from the raw data file. In some instances, the Idylla reports only the mutation that was predominantly detected. More than two co-existing mutations may not be detected or visible in the raw data at all. We have seen several cases in which the raw data of the Idylla suggested the presence of two independent deletions in exon 19. Orthogonal testing with NGS, however, confirmed only one of these mutations. The Idylla report displayed only “Exon 19 deletion” without further

specification of the number or characterization of the underlying exon 19 deletion(s). It has also been reported that variants nearby Idylla panel mutations, or two DNA changes located in the same or in two consecutive codons, and indel mutations at a location in which the Idylla only identifies a single nucleotide variant, can lead to erroneous calls (7, 8, 19, 20). We have not encountered such incidences. However, we have observed cross-reactivities in the presence of two independent mutations in one gene, mostly but not always one of them being an *EGFR* deletion in exon 19 (Figure 3). For example, in the presence of a *KRAS* G13D mutation, cross-reactivity with the G12V mutation resulting in a G12V positive call is well described. The filtering algorithm of the Biocartis software will not always detect these errors. However, when comparing Cq and delta Cq values and the amplification curves of the PCR reaction, cross-reactivities can usually be identified.

Without consulting the raw data, mutations can also be missed easily if the DNA input was sufficient but low - indicated by a high Cq value (commonly >30) . Despite a 'valid run,' the presence of a mutations may not display on the console report or on the curve report. The specific thresholds for the sufficient DNA amount and their respective Cq values have not been published by the manufacturer and could not be established in this study due to limited sample availability.

4. Discussion

Despite the agreement on the importance of molecular biomarker assessment, molecular testing is still not performed in many cancer patients (21, 22). Delaying treatment decisions for weeks until test results are available is not an option for many patients. The Idylla mutation assay is a rapid test that has only 2-3 hours runtime. However, the time from obtaining the specimen in the operation room to result release still takes a week or longer. This warrants careful reassessment of internal workflows to identify steps that could improve overall turnaround time and make the Idylla worthwhile. Attempts have been made by other groups by taking touch preparations from resected tumor tissue right in the operation room and moving them straight into molecular testing (23). Although this approach provides molecular results much faster, in many cases, a diagnosis is still pending and it is unclear which test to perform. A major challenge of the Idylla mutation test is the lack of tissue available for testing. In many instances the available tissue needs to be used first for diagnostic tests that establish the diagnosis. Each Idylla cartridge uses a fair amount of tissue. In particular when several genes are to be tested, the one-gene-one-cartridge approach of the Idylla can become challenging or make its use unattainable.

Many studies demonstrated the high accuracy of the Idylla mutation test in a range of 95-100% (7, 8, 19, 20, 24, 25). The 100% sensitivity and specificity that we obtained in our validation out-performed our expectations. However, the sample size and selection for our validation pool were carefully designed to represent our patient population and are comparable to other studies (19, 20). A larger sample size or inclusion of different mutations may not have yielded a perfect validation result. Several groups have reported false negative results with the Idylla (7, 8, 19, 20). These instances were attributed to low tumor cell content in the sample that does not meet the minimum requirements of the assay (Supplemental Table 5). For the Idylla mutation test, the

subjective evaluation of exceeding the threshold for tumor cell content in a background of normal cells can be challenging. The usual parameters for molecular tests, such as DNA quality and quantity cannot be determined for the Idylla mutation test. We therefore recommend using it only on specimens that clearly show a tumor cell content above the required minimum. For the *BRAF* cartridge, a neoplastic cell content of ≥50% in the test sample is recommended by the manufacturer, unless macro-dissection is performed. However, in many instances the neoplastic cell content is less, and macro-dissection is not performed in all laboratories. Therefore, the current *BRAF only* cartridge has limited utility for clinical samples. A more recent Biocartis product, the combined *NRAS-BRAF* cartridge requires a tumor cell content of only 10% and therefore may be more suitable for clinical testing. In the context of false positive and negative results, it is worth mentioning that we have identified mutations with the Idylla that were not detected by NGS that had a detection limit of 5% allele frequency. Not only false negative, also false positive results were reported with the Idylla. They are thought to occur mainly due to cross-reactivities between certain gene regions (see 3.5. Challenges with result interpretation).

For mutations in *EGFR*, the Idylla reports 36 genuine DNA changes cumulatively as 'Exon 19 deletion.' So far, the distinction between these actual DNA changes has not been clinically relevant. As we learn more about genotype-phenotype interactions, this could change. Zhao at al. reported different subtypes of patients based on their specific *EGFR* exon 19 deletion. Patients may have different clinico-pathological features, different resistance patterns when treated with first-line TKIs, or significantly shorter progression-free survival (13). We likely will soon need to understand the specific underlying DNA changes for a more refined interpretation, and targeted treatment approach. The Idylla *BRAF* cartridge demonstrates this trend already on which V600E/D and V600K/R/M are discerned. According to current guidelines of the National

Comprehensive Cancer Network (NCCN), only *BRAF* V600E and K are considered for treatment in the United States (26, 27). Although the Idylla detects *BRAF* V600E and K if present, from the Idylla result output it cannot be inferred which of the five amino acid substitutions is actually present in the sample. Therefore, treatment justification and approval may pose challenges.

Current NCCN guidelines for NSCLC recommend treatment strategies for *EGFR* exon 20 insertions (1, 2). The Idylla assay detects four different exon 20 insertions, but many more exon 20 insertions exist. Most lung cancers with exon 20 insertions are unresponsive to TKI therapy. Well known examples are the mutations A763_Y764insFQEA and A763_Y764LQEA (1, 2, 28-30) which are both recognized in the NCCN guidelines. However, both are not included on the current Idylla panel. It is critical yet challenging that mutation panels stay current to meet the needs for diagnostic testing.

Considering future trends, the potential impact of intra-tumor heterogeneity, clonality, and variant allele fraction and the uneven distribution of mutation across tumor tissue, which can be far below 1%, are increasingly recognized for its diagnostic importance (31, 32). The Idylla mutation test is a qualitative test that, at its present stage, does not inform about any of these aspects.

5. Conclusions

The Idylla mutation test has been a significant breakthrough for the fully-automated and rapid detection of cancer mutations. With appropriate quality control measures in place, the test delivers fast, cost effective and trustworthy results, avoiding the need for expensive and time

consuming NGS analyses in many cases. We recommend the Idylla mutation test as a first tier screening test for which negative results should be followed with additional testing by NGS. Limitations in the current assay design may impact clinical utility as the field of molecular diagnostics is moving forward. The increasing need for expanded mutation testing, explicit result reporting, and information about variant allele frequencies are important aspects to consider for future development.

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433

Figure 1. Idylla Workflow. Intradepartmental workflow demonstrating the sample processing from surgical retrieval to result reporting.

Figure 2. Sample processing time. Sample processing time improved over time. However, due to the sequential process of the workflow, the overall turnaround time is (TAT) are significantly longer than the actual assay run time.

Figure 3. False positive double mutation due to cross-reactivities. The mutation 'Del 9/21a' called by the Idylla in cartridge chamber B was confirmed with NGS as c.2239_2248delinsC (p.Leu747_Ala750delinsPro) at the allele frequency of 6%. The 'Del12' identified by the Idylla in chamber C of the same cartridge was not confirmed by NGS.

Authors' Disclosures or Potential Conflicts of Interest:

Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest: Employment or Leadership: None declared. Consultant or Advisory Role: None declared. Stock Ownership: None declared. Honoraria: None declared. Research Funding: None declared. Expert Testimony: None declared. Patents: None declared.

Table 1. Clinical test orders and mutation spectrum. The Idylla orders placed from October 2020 - March 2022 were predominantly for patients with lung cancer. The most frequently used cartridges were for *EGFR* and *BRAF* testing. Due to limited shelf life of the cartridges, timely planning for cartridge orders is important.

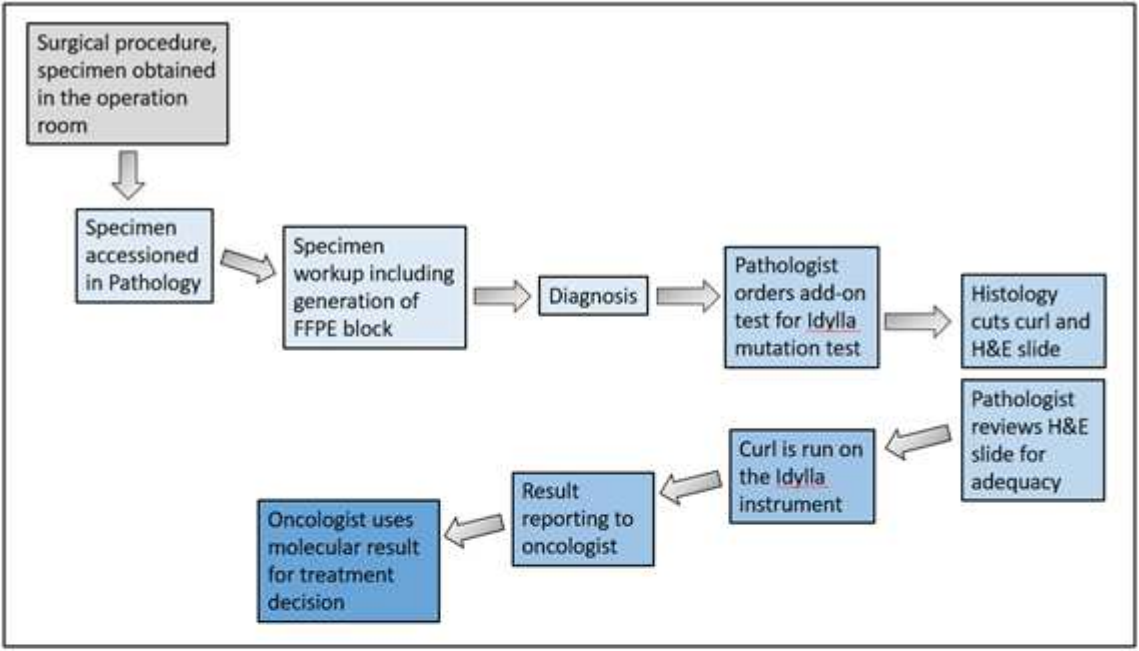
Diagnosis		NSCLC	Lung cancer, other	Colorectal Cancer	Melanoma	Other cancer	Total number of cartridges	Specific mutation detected	Number of observations
Number of patients		80	4	14	6	11			
Gene tested	*EGFR*	●	●			○	89	**Exon 19 deletion**	9
								L858R	8
								Exon 20 insertion	1
								L861Q	1
								G719A/C/S	1
	BRAF (on BRAF only and combined NRAS/BRAF cartridge)	○		●	●	●	30	**V600E/D**	7
								V600K/R/M	1
	KRAS			●		○	19	**G12D**	6
								G12C	1
								G12S	1
	NRAS			○		○	11	Q61L	1

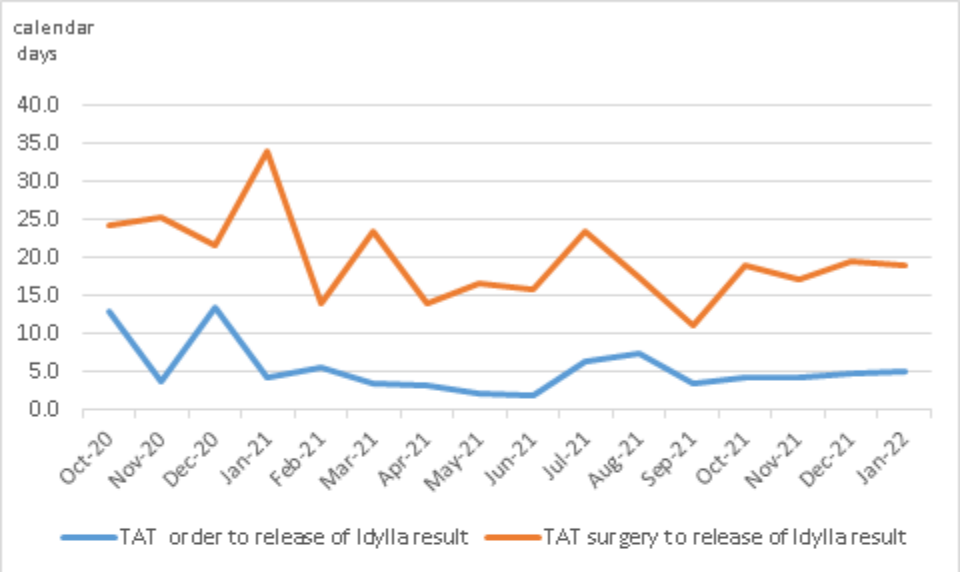
● frequently ordered test

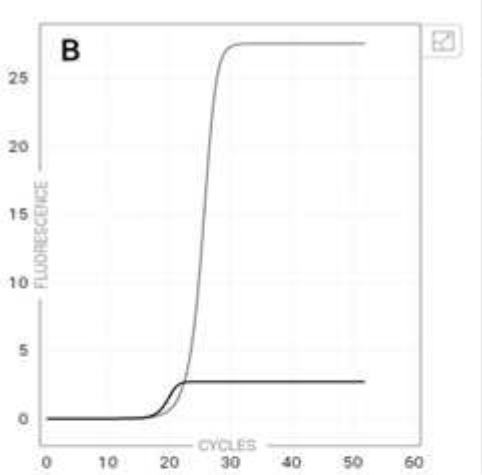
○ infrequently ordered test

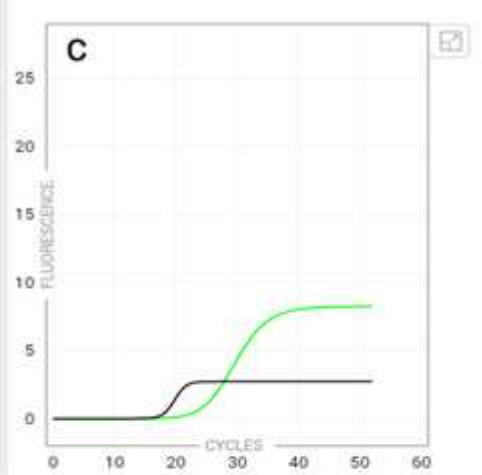
Table 2. Test order algorithm at TMC. In NSCLC, only resection material from tumors stage 1-3 is used for testing with the Idylla. For cutaneous melanoma, the Idylla mutation test is only applied in critically ill patients for which immediate treatment decisions are crucial.

Lung cancer	Biopsy	Stage 1-4	Preserve tissue	Send out to reference lab, NGS gene panel
	Resection	Stage 1-3	*EGFR (KRAS)*	Idylla mutation test
		Stage 4	Preserve tissue	Send out to reference lab, NGS gene panel
Colorectal cancer	Biopsy or resection	Any stage	*KRAS* reflex to *BRAF* (and *NRAS*)	Idylla mutation test
Cutaneous melanoma	Biopsy or resection	Critically ill patients	*BRAF* (*NRAS*)	Idylla mutation test


Surgical procedure, specimen obtained in the operation room
Specimen accessioned in Pathology
Specimen workup including generation of FFPE block
Diagnosis
Pathologist orders add-on test for Idylla mutation test
Histology cuts curl and H&E slide
Pathologist reviews H&E slide for adequacy
Curl is run on the Idylla instrument
Result reporting to oncologist
Oncologist uses molecular result for treatment decision


calendar days
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Oct-20
Nov-20
Dec-20
Jan-21
Feb-21
Mar-21
Apr-21
May-21
Jun-21
Jul-21
Aug-21
Sep-21
Oct-21
Nov-21
Dec-21
Jan-22
TAT order to release of Idylla result
TAT surgery to release of Idylla result





ED

TARGET	CQ	ΔCQ
Del 9/21a	24.16	5.45
EGFR Total	18.71	-

TARGET	CQ	ΔCQ
Del 12	26.90	8.14
EGFR Total	18.77	-

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalData.docx