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Multi-center evaluation of the novel fully-automated PCR-based Idylla™ BRAF Mutation Test on formalin-fixed paraffin-embedded tissue of malignant melanoma

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ABSTRACT

The advent of BRAF-targeted therapies led to increased survival in patients with metastatic melanomas harboring a BRAF V600 mutation (implicated in 46–48% of malignant melanomas). The Idylla™ System (Idylla™), i.e., the real-time-PCR-based Idylla™ BRAF Mutation Test performed on the fully-automated Idylla™ platform, enables detection of the most frequent BRAF V600 mutations (V600E/E2/D, V600K/R/M) in tumor material within approximately 90 min and with 1% detection limit.

Idylla™ performance was determined in a multi-center study by analyzing *BRAF* mutational status of 148 archival formalin-fixed paraffin-embedded (FFPE) tumor samples from malignant melanoma patients, and comparing Idylla™ results with assessments made by commercial or in-house routine diagnostic methods.

Of the 148 samples analyzed, Idylla™ initially recorded 7 insufficient DNA input calls and 15 results discordant with routine method results. Further analysis learned that the quality of 8 samples was insufficient for Idylla™ testing, 1 sample had an invalid routine test result, and Idylla™ results were confirmed in 10 samples. Hence, Idylla™ identified all mutations present, including 7 not identified by routine methods.

Idylla™ enables fully automated BRAF V600 testing directly on FFPE tumor tissue with increased sensitivity, ease-of-use, and much shorter turnaround time compared to existing diagnostic tests, making it a tool for rapid, simple and highly reliable analysis of therapeutically relevant BRAF mutations, in particular for diagnostic units without molecular expertise and infrastructure.

1. Introduction

v-Raf murine sarcoma viral oncogene homolog B (BRAF) belongs to the Raf family of serine/threonine-specific protein kinases, which activate the RAF–MEK–ERK (MAPK) pathway and are crucial in the proper maintenance of cell growth, differentiation, and survival (Chong et al., 2003). The Raf family comprises three members: A-Raf, BRAF, and C-Raf. Somatic missense mutations in the gene encoding the BRAF protein (*BRAF*: NM 004333), are implicated in 46–48% of malignant melanomas, 100% of hairy cell leukemia, 44% of papillary thyroid cancers, and 8–15% of colorectal cancers, and in many other human cancers at lower frequencies (Davies et al., 2002; Hall and Kudchadkar, 2014). The most common *BRAF* mutation (73%) in metastatic melanoma is a T to A base transversion at nucleotide position c.1799 in exon 15 encoding the tyrosine kinase domain, which results in a valine to glutamate substitution at position 600 of the protein (V600E) (Menzies et al., 2012). The remaining *BRAF* mutations are primarily GT

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to AA transversions at nucleotide position c.1798–1799 (19%) resulting in a valine to lysine substitution at codon 600 (V600K). Less-frequent base changes such as TG to AA (V600E2), TG to AT (V600D), and TG to AC (V600D) at nucleotide position c.1799–1800, as well as a GT to AG transversion at nucleotide position c.1798–1799 (V600R), and G to A at nucleotide position c.1798 (V600M), have also been reported.

The annual incidence of malignant melanoma has increased dramatically over the past few decades, with approximately 80,000 and 100,000 new cases of melanoma being registered yearly in the US and Europe, respectively (Ferlay et al., 2013; Siegel et al., 2015). Surgical resection is able to cure primary cutaneous melanoma, but metastatic disease proves to be fatal with median survival for stage IV melanoma patients ranging from 8 to 18 months after diagnosis (Balch et al., 2009). The advent of BRAF-targeted therapies, such as the selective V600-mutated BRAF inhibitors vemurafenib and dabrafenib, alone or in combination with MEK inhibitors, led to increased survival in patients with the BRAF V600E or V600K mutation (Spagnolo et al., 2015). There is clinical evidence suggesting that also patients harboring the less common V600D, V600R, or V600M mutations may be treated successfully with vemurafenib or dabrafenib (Klein et al., 2013; Parakh et al., 2015; Ponti et al., 2013).

According to the European Society for Medical Oncology (ESMO), genotyping of the *BRAF* V600 mutational status is mandatory for metastatic melanoma patients. Several tests analyzing the *BRAF* mutational status are available. These tests include Sanger sequencing, pyrosequencing, next generation sequencing, immunohistochemistry, and tests based on real-time polymerase chain reaction (PCR), with each method having its own sensitivity, specificity, cost, and turnaround time (Colomba et al., 2013; Ihle et al., 2014). Sanger sequencing has for many years been considered the standard method for the detection of mutations from tumor DNA, but is hampered by low sensitivity. Immunohistochemistry is cheap and results are obtained within 48 h, however the technique is highly specific for V600E and missing all other BRAF mutations. Pyrosequencing is highly sensitive although prone to errors without using customer-designed set-up. Real-time-PCR-based strategies are more sensitive compared to Sanger, but commercial tests specifically designed for detecting V600E miss some of the less common V600 mutations. For example, the cobas® 4800 BRAF V600 Mutation Test (Roche, Basel, Switzerland) shows only limited cross-reaction with V600E2 (≥65% mutation), V600K (≥35% mutation), and V600D (≥10% mutation), and the therascreen BRAF RGQ PCR Kit (Qiagen, Venlo, The Netherlands) detects V600E, V600D, V600K, and V600R, but misses V600M. Next generation sequencing has high sensitivity and multiplexing options, allowing generation of a molecular profile of each tumor sample analyzed, but on the down side this technique is expensive and time consuming and generates a lot of information while only the *BRAF* mutational status is of interest (Colomba et al., 2013; Ihle et al., 2014). In general, current diagnostic tests for *BRAF* mutational status are labor-intensive requiring specialized staff and a complex infrastructure. They are therefore often performed by a third party, which delays therapeutic decisions.

The Idylla™ BRAF Mutation Test performed on the Idylla™ molecular diagnostics platform (Biocartis, Mechelen, Belgium) recently received CE-IVD certification for the detection of BRAF mutations in formalin-fixed paraffin-embedded (FFPE) material of malignant melanoma. It is a fully-automated real-time-PCR-based test designed for the qualitative detection of the V600E/E2/D and V600K/R/M mutations, with high sensitivity (detection of 1% mutant in a background of wild type) and quick turnaround time (approximately 90 min) from FFPE sample to final result (Janku et al., 2015; Schiefer et al., 2015). The single-use cartridge-based Idylla™ BRAF Mutation Test is performed directly on FFPE tissue sections, requiring no beforehand sample preparation and minimal hands-on time (less than 2 min per FFPE tissue section). The interpretation of results is fully automatic. Given the characteristics of the platform, the test can easily be performed on-site without needing extra-molecular infrastructure or a highly skilled staff.

In the current multi-center study, archival FFPE tumor samples from melanoma patients were tested for *BRAF* mutational status using the Idylla™ BRAF Mutation Test. To establish test performance, the results were compared to the results previously obtained with routine reference methods.

2. Materials and methods

2.1. Samples

In the current multi-center study, a total of 148 clinical leftover FFPE samples of human melanoma were retrospectively analyzed for *BRAF* mutational status using the Idylla™ BRAF Mutation Test on the Idylla™ platform. The *BRAF* mutational status of these samples was assessed previously to guide cancer treatment, by Sanger sequencing, pyrosequencing, or PCR-based methods (commercial and in-house). The results, obtained using the Idylla™ BRAF Mutation Test on archival material, were not used for diagnostic purposes of any kind. Samples were tested at six centers: Hospital del Mar (Barcelona, Spain; n = 32), Imelda Hospital (Bonheiden, Belgium; n = 24), Copenhagen University Hospital (Copenhagen, Denmark; n = 29), Laboratorium Pathologie Oost-Nederland (Hengelo, The Netherlands; n = 22), Jan. Yperman Hospital (Ieper, Belgium; n = 14), and Städtischen Klinikum Karlsruhe (Germany; n = 27).

In the current study, a number (i.e., mostly 1 or 2, up to 9) of 5 μm to 40 μm thick FFPE tissue sections were sampled as close as possible (within the FFPE block) to the tissue sections used to generate the reference result (see Supplementary Table 1). Before the analyses started, the tumor content and area was determined on a hematoxylin–eosin (HE)-stained slide by a Pathologist. Based on the visual assessment of the HE staining of consecutive slides, appropriate macrodissection was performed before loading the cartridge to achieve a tumor cell content of at least 50%. These selected FFPE tissue sections were placed directly into the Idylla™ cartridge following the instructions for use. In each of the six study centers, two artificial FFPE samples (i.e., BRAF V600E and BRAF V600K FFPE Reference Standards; Horizon Diagnostics, Cambridge, UK) were included as external controls.

2.2. Idylla™ System

The Idylla™ molecular diagnostics platform (Biocartis, Mechelen, Belgium) is a random-access fully automated system providing sample to result functionality, based on allele-specific real-time PCR. The instrument covers fully integrated sample preparation combined with PCR thermocycling and fluorescence detection of target sequences. Disposable cartridges will enable the simultaneous detection of up to 30 molecular targets from a variety of solid and liquid sample types, including FFPE slides and slices, swab, blood, urine, stool, sputum, and tissue. For FFPE material, the sample preparation module uses high-frequency intensity

Table 1
Comparison between results of Idylla™ and of routine reference methods.

		Routine reference method including further analysis[a]				
		V600E or D	V600K, R or M	No mutation	Insufficient DNA input	Total
Idylla™	V600E/E2/D	**67**	1	1	1	70
	V600K/R/M	2	**10**	8	0	20
	No mutation	1	1	**49**	0	51
	Insufficient DNA input	1	0	6	0	7
	Total	71	12	64	1	148

Bold means 'concordant results'.

[a] Different routine reference methods were used: cobas® 4800 BRAF V600 Mutation Test (Roche), BRAF RGQ PCR Kit (Qiagen), BRAF StripAssay® V600E (Viennalab), Sanger sequencing, and several in-house BRAF tests (based on high-resolution melting, allele-specific and wild-type blocking real-time PCR).

focused ultrasound (HiFU) technology in combination with buffers to emulsify the paraffin while simultaneously re-hydrating the tissue sample in an aqueous solution, leading to DNA liberation. The test does not require prior manual deparaffinization or FFPE pre-processing. Via microfluidic channels in the cartridge, nucleic acids are transported into 5 separate PCR chambers, which contain pre-deposited PCR reagents in dried form (i.e., primers, probes, enzymes) for the simultaneous analysis of the sample DNA and an endogenous sample processing control. Every PCR chamber enables the identification of up to 6 different targets (amounting to 30 groups of markers), each of which can be composed of multiple individual biomarkers. Detection of these specific targets is performed using fluorescently labeled probes. All consumables required to perform sample preparation and real-time PCR detection are provided in the cartridge. Closing of the cartridge after inserting the sample eliminates the possibility of cross contamination between different samples.

2.3. Idylla™ BRAF Mutation Test

The Biocartis' Idylla™ BRAF Mutation Test is a single-use cartridge-based test designed for the qualitative detection of the V600E/E2/D and V600K/R/M mutations in codon 600 of the *BRAF* gene. The test consists of 3 allele-specific duplex PCR reactions (in 3 PCR chambers), designed to specifically amplify either the *BRAF* wild type, *BRAF* c.1799T>A, and *BRAF* c.1798G>A nucleotide changes, and hence does not discriminate between mutations leading to BRAF V600E, E2, and D changes (V600E/E2/D; all c.1799T>A) on the one hand, and V600K, R, and M mutations (V600K/R/M; all c.1798G>A) on the other hand. The analytic time required to perform the test up to result reporting is approximately 90 min, with a hands-on time of less than 2 min. The test enables identifying the presence of ≥1% BRAF V600 mutation in a background of wild-type allele.

2.4. Analysis of collected fluorescent signals

A quantification cycle value (Cq) value is calculated by Idylla™ software for every valid PCR curve. The presence of a mutant genotype is determined by calculating the ΔCq, i.e., the difference between the BRAF wild type Cq and the V600E/E2/D or V600K/R/M Cq values. The mutant signal is considered valid if the ΔCq is within a validated range, and the sample will then be characterized as BRAF V600 mutation positive, indicating the specific mutation group. All samples with a valid wild-type signal but a ΔCq value outside the validated range are characterized as BRAF V600 mutation negative.

2.5. Sanger sequencing

Sanger sequencing was used as a routine reference method for a number of samples, or was used to further analyze some of the samples having an Idylla™ BRAF Mutation Test result not concordant with the result of the routine reference method. Briefly, after extraction of genomic DNA from FFPE slices, the dideoxynucleotide sequencing of *BRAF* exon 15 was performed in nested PCR reactions using AmpliTaqGold® (Life Technologies, Thermo Fisher Scientific, Waltham, MA) or RedEx PCR master mix (Sigma-Aldrich, St. Louis, MO), and specific primers. PCR products were purified with the MultiScreen® PCRμ96 Filter Plate (MerckMillipore, Billerica, MA) or the QIAquick PCR® purification kit (Qiagen), and sequenced with BigDye® Terminator 3.1 Cycle Sequencing Kit (Life Technologies) on an Applied Biosystems® 3500 Dx DNA sequenator (Life Technologies) according to the manufacturer's instructions.

2.6. Commercial routine BRAF mutation tests used as reference method

The cobas® 4800 BRAF V600 Mutation Test (Roche), BRAF RGQ PCR Kit (Qiagen), and BRAF StripAssay® V600E (Viennalab, Vienna, Austria) were performed according to the manufacturer's instructions.

2.7. In-house routine BRAF mutation tests used as reference method

Wild-type blocking real-time PCR was performed on a LightCycler® 480 real-time PCR instrument (Roche) using LightCycler® 480 SYBR Green (Roche) to detect DNA amplification. Allele-specific real-time PCR was performed using dual-labeled probes on a cobas® Z480 Analyzer (Roche) essentially as described before (van Eijk et al., 2011). Digital droplet PCR was performed on a QX100™ system (Bio-Rad Laboratories, Hercules, CA) according to the instructions of the manufacturer. High resolution melting analysis was performed on a LightCycler® 480 (Roche Diagnostics, Vienna, Austria), with primers flanking a 177 bp amplicon of BRAF exon 15 encompassing the V600 codon; analysis of the melting curves was performed using the LightCycler® 480 Gene Scanning Software (Roche).

3. Results

3.1. PCR curves

Representative examples of PCR curves and the corresponding Cq values for different Idylla™ analysis results are shown in Fig. 1.

3.2. Results of Idylla™ BRAF Mutation Test

The *BRAF* mutational status of 148 retrospective clinical FFPE samples of various tissues from patients with melanoma was tested at six centers with the Idylla™ System (Idylla™), i.e., the BRAF Mutation Test performed on the Idylla™ platform, and the results were compared with the original assessments made by several routine reference methods (Supplementary Table 1). Idylla™ results were not used for any diagnostic purposes.

The following routine reference methods were used: cobas® 4800 BRAF V600 Mutation Test (Roche; n = 40; the test result did not specify the exact nature of the detected mutation, but, as this test was designed to detect V600E, mutations detected were considered V600E for the analysis), BRAF RGQ PCR Kit (Qiagen; n = 28), BRAF StripAssay® V600E (Viennalab; n = 1), Sanger sequencing (as only method n = 29, combined with other method n = 32), wild-type blocking real-time PCR (in house; n = 14), allele-specific real-time PCR using dual-labeled probes (in house; n = 15), real-time PCR and high resolution melting (HRM) analysis (in house; n = 22). To maximize sensitivity, tumor area was enriched by macrodissection in 79 cases. In 1 case, microdissection was applied after removal of paraffin and HE staining of the sections. Samples in which no tumor tissue was observed by staining and visual control were excluded from the data set.

Of the 148 FFPE samples analyzed, 70 tested positive for a V600E/E2/D mutation and 20 tested positive for V600K/R/M on Idylla™, in 51 cases no mutation was found, and Idylla™ reported insufficient DNA input for 7 samples (Table 1). Overall in the first assessment, Idylla™ demonstrated agreement with the routine reference methods in 126 samples. In 9 samples Idylla™ detected a mutation while no mutation was detected by the reference method, and in 2 cases Idylla™ did not detect the mutation observed by a reference method. In 3 samples, different mutations were detected. The routine reference methods detected 71 V600E, 10 V600K, 1 V600R, and 1 V600M mutation.

The 7 insufficient DNA input calls and 15 discordant samples were further analyzed.

3.3. Insufficient DNA input call by Idylla™ BRAF Mutation Test

Insufficient DNA input was reported by Idylla™ in 7 samples (Table 2). The Idylla™ BRAF Mutation Test requires a minimal tissue area of the specimen of 50 mm^2 when 5 μm tissue sections are used

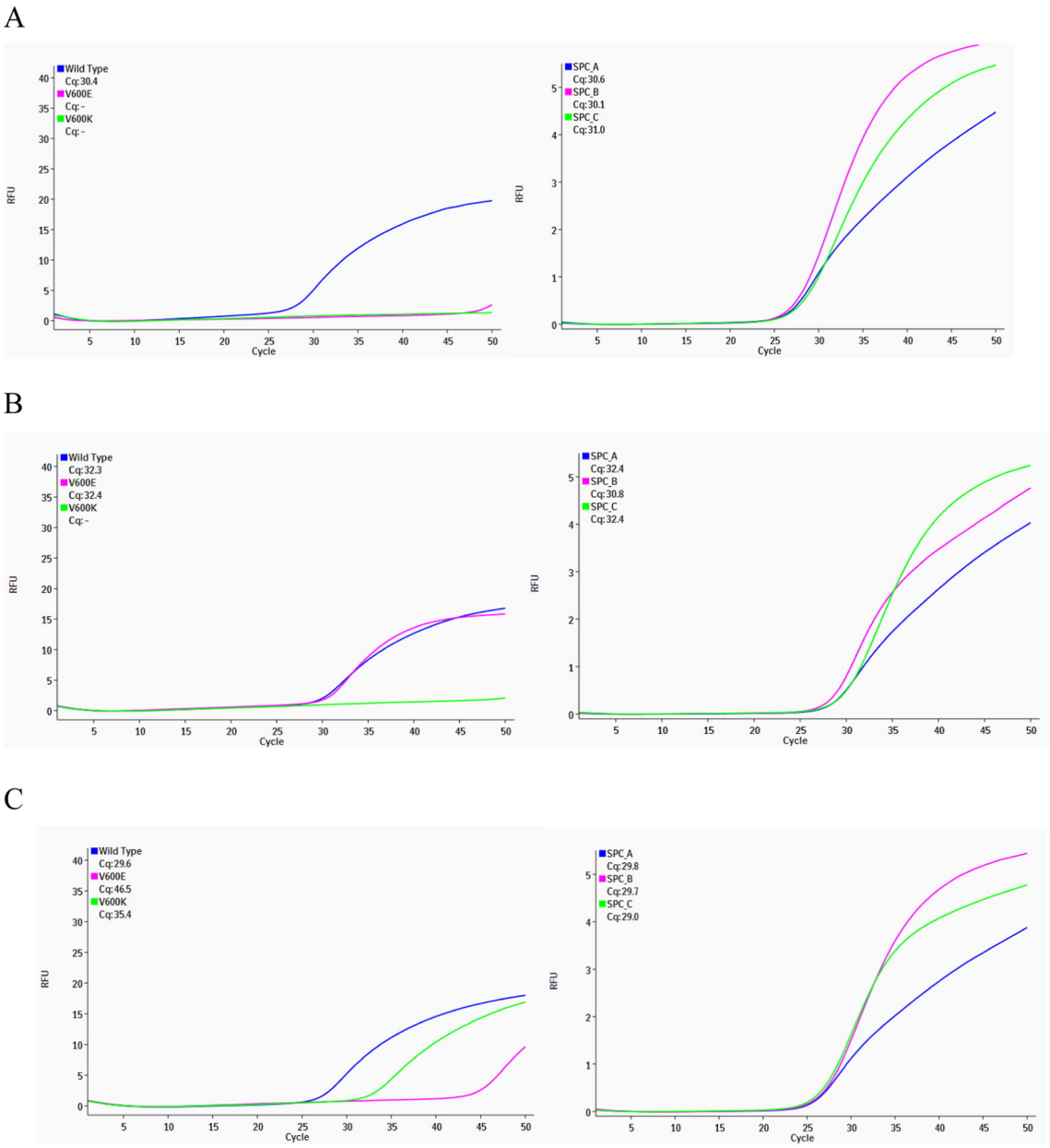

Fig. 1. Representative examples of PCR curves for samples (left panels) and sample processing controls (SPC_A, SPC_B, and SPC_C; right panels) for the different Idylla™ analysis results: (A) no mutation detected call; (B) V600E/E2/D mutation detected call; and (C) V600K/R/M mutation detected call.

and of 25 mm^2 when using 10 μm tissue sections, with a minimum tumor cell percentage of 50%.

For Sample 3, a tumor area of 375 mm^2 (for two 40 μm tissue sections) was analyzed, which is more than 10 times the maximum tissue area as specified in the Idylla™ instructions for use. Idylla™ retesting of this FFPE sample with less material confirmed the mutation previously found with Sanger sequencing. Hence, it is conceivable that the first test run failed due to an excess of DNA, paraffin, and melanin.

Samples 19, 23, 52, 118, and 148 did not meet the minimum requirements for Idylla™ testing. Retesting of Sample 23 by Idylla™ did again result in an insufficient DNA input call. Sample 118 contained a very small tissue fragment and visual inspection showed that there was little tissue left in the FFPE block. For sample 50, the tumor area was not determined; hence it is unknown if the minimum requirements for Idylla™ testing were met.

3.4. Discordances between Idylla™ BRAF Mutation Test and routine reference methods

For 15 samples, discordant results were found by Idylla™ as compared to the previous results of routine reference methods (Table 3). Idylla™ detected another mutation as compared to the one detected by the reference method in 3 samples, and detected a mutation while the reference method did not find a mutation in 10 samples (including 1 sample where the reference method failed due to insufficient DNA input). In 2 samples, the mutation detected by the reference method was not found by Idylla™.

The Idylla™ results were confirmed by a third method in Samples 35, 45, and 10, (digital droplet PCR), and 59, 62, 66, 68, and 76 (Sanger sequencing). Concerning Samples 72 and 77, the cobas® 4800 BRAF V600 Mutation Test (Roche) identified a mutation, which most probably is V600E, but might be another V600 mutation as well. As the

Table 2
Insufficient DNA input call by Idylla™.

Sample #	Tissue type	FFPE tissue section (μm)	Number of FFPE tissue sections	Tumor cells (%)	Tumor area (mm^2)	Macrodissection	Idylla™	Routine reference method	Further analysis
3	Skin	40	2	>85	375	Yes	Insufficient DNA input	V600E[a]	V600E[e]
19	Liver	20	1	>80	6	No	Insufficient DNA input	No mutation[a]	Insufficient sample quality
23	Skin	40	2	>80	2	Yes	Insufficient DNA input	No mutation[a]	Insufficient sample quality
52	Skin forehead	5	3	/	<50		Insufficient DNA input	No mutation[b]	Insufficient sample quality
118	Skin thigh	5	6	70	30	Yes	Insufficient DNA input	No mutation[c]	Insufficient sample quality
148	Thyroid	10	1	20	36		Insufficient DNA Input	No mutation[d]	Insufficient sample quality
50	Liver	5	3	>90	ND	No	Insufficient DNA input	No mutation[b]	ND

ND, not determined.
[a] Sanger sequencing.
[b] Wild-type blocking real-time PCR (in house).
[c] Allele-specific real-time PCR (in house).
[d] BRAF RGQ PCR Kit (Qiagen).
[e] Idylla™.

presence of a V600 mutation is sufficient to start BRAF-targeted therapy, the exact nature of the mutation identified by the reference method was not determined. Hence, the Idylla™ result might be in concordance with the reference method result, but discordance cannot be ruled out. Sample 123 was shown to be cross-contaminated and Idylla™ retesting of the uncontaminated sample confirmed the no mutation finding of the routine reference method. Digital droplet PCR did not confirm the Idylla™ result for Sample 115, but further examination of the FFPE block by HE staining showed bad fixation of the tissue and uncertainty about the presence of tumor tissue.

No mutation was detected by Idylla™ in Samples 34 and 36, while the routine reference method found V600E and V600M, respectively. A new HE staining was performed on the last slice of both FFPE blocks. In Sample 34, no tumor tissue was found. For Sample 36 the new staining confirmed tumor cells and area, but less than 50% of the sample consisted of tumor cells, which is below the minimal requirement for Idylla™ testing, and these were scattered between the normal cells.

In Sample 40, Idylla™ found V600E/E2/D, but the routine reference method failed due to insufficient DNA input.

3.5. Performance of the Idylla™ BRAF Mutation Test

Further analysis of the Idylla™ insufficient DNA input calls, resulted in 1 additional sample (Sample 3) where Idylla™ and routine reference method results were in agreement. As to the discordant results, Idylla™ findings were confirmed by a third method in 8 samples (Samples 35, 45, 10, 59, 62, 66, 68, and 76), and cross-contamination led to a discordant result in 1 sample (Sample 123), which could be corrected. Hence, in 10 more samples Idylla™ was found to have results concordant with the confirmed test results.

On the other hand, 5 samples having an Idylla™ insufficient DNA input call did not meet the minimal requirements for Idylla™ testing. In 2 samples (Samples 34 and 36) where Idylla™ reported no mutation but the routine reference method did, too little tumor tissue was present for valid Idylla™ testing. In 1 sample (Sample 115) where Idylla™ reported a mutation which was not detected by the reference method, the quality of the FFPE block was insufficient. Finally for 1 sample (Sample 40), no reference method result was available. Therefore, 9 samples were removed from the dataset.

Table 3
Discordant results between Idylla™ and routine reference methods.

Sample #	Tissue type	FFPE tissue section (μm)	Number of FFPE tissue sections	Tumor cells (%)	Tumor area (mm^2)	Macrodissection	Idylla™	Routine reference method	Further analysis
35	Skin	5	6	>90	35	Yes	V600E/E2/D	V600K[a]	V600E[f]
45	lymph node	10	3	40	28	Yes	V600E/E2/D	No mutation[b]	V600E[f]
10	Skin	40	2	>80	3	Yes	V600K/R/M	No mutation[c]	V600K[f]
59	Lymph node	5	1	90	180	No	V600K/R/M	No mutation[d]	V600K[c]
62	Muscle	5	1	90	145	No	V600K/R/M	No mutation[d]	V600K[c]
66	Skin	5	1	80	50	No	V600K/R/M	No mutation[d]	V600K[c]
68	Skin	5	4	40	60	No	V600K/R/M	No mutation[d]	V600K[c]
76	Lymph node	5	1	40	32	No	V600K/R/M	No mutation[d]	V600K[c]
72	Skin	5	1	80	77	No	V600K/R/M	Mutation[d]	ND
77	Skin	5	2	30	55	Yes	V600K/R/M	Mutation[d]	ND
123	Skin	?	1	70	130		V600K/R/M	No mutation[e]	No mutation[g]
115	Skin upper arm	5	4	70	80	Yes	V600K/R/M	No mutation[d]	Insufficient sample quality
34	Melanoma	Gland	5	6	>90	Yes	No mutation	V600E[a]	Insufficient sample quality
36	Peritoneum	5	4	<50	70	Yes	No mutation	V600M[a]	Insufficient sample quality
40	Skin	5	6	>90	6	Yes	V600E/E2/D	Insufficient DNA input[a]	ND

ND, not determined.
[a] Wild-type blocking real-time PCR (in house).
[b] Allele-specific real-time PCR using dual-labeled probes (in house).
[c] Sanger sequencing.
[d] cobas® 4800 BRAF V600 Mutation Test (Roche); test result did not specify exact nature of detected mutation.
[e] BRAF RGQ PCR Kit (Qiagen).
[f] Digital droplet PCR.
[g] Idylla™.

Table 4
Performance of Idylla™.

		Routine reference method including further analysis[a]				
		V600E or D	V600K, R or M	No mutation	Insufficient DNA input	Total
Idylla™	V600E/E2/D	**70**	0	0	0	70
	V600K/R/M	2	**16**	0	0	18
	No mutation	0	0	**50**	0	50
	Insufficient DNA input	0	0	1	0	1
	Total	72	16	51	0	139

Bold means 'concordant results'.
[a] Including routine reference tests and confirmation tests, different methods were used: cobas® 4800 BRAF V600 Mutation Test (Roche), BRAF RGQ PCR Kit (Qiagen), BRAF StripAssay® V600E (Viennalab), Sanger sequencing, digital droplet PCR, and several in-house BRAF tests (based on high-resolution melting, allele-specific and wild-type blocking real-time PCR).

Taking into account the additional concordant results and the samples removed from the dataset, Idylla™ results were in agreement with the confirmed test results from reference methods in 136 of 139 samples, resulting in an overall concordance of 97.84% (Table 4).

Overall in the clinical FFPE samples, Idylla™ identified all the V600E or V600K mutations found by routine reference methods. In addition (as established by further analysis, see Table 3), Idylla™ found a mutation where the routine method did not in 7 cases, and identified a mutation different from the routine test result in 1 case.

4. Discussion

During the last decade, the field of oncology has experienced large changes with the cancer biomarkers and personalized cancer therapy. Patient-personalized cancer therapies are now available for, e.g., lung cancer patients with mutated *EGFR* (erlotinib, gefitinib, and afatinib), colorectal cancer with wild type *KRAS* and *NRAS* (cetuximab and panitumumab) and metastatic melanoma with mutated *BRAF* (vemurafenib and dabrafenib). Modern pathology analysis requires often a number of immunohistochemical as well as molecular biological tests on often small biopsies and samples with low content of tumor material. In contrast to a constantly increasing number of treatment-relevant biomarkers is a rising demand for fast clinical investigation and disease sub-classification in order to assign the patient to the most appropriate treatment regimes.

Since the approval of vemurafenib in 2012, assessment of BRAF codon 600 mutation status has been mandatory prior to treatment of patients with advanced or metastatic melanomas. An important feature of selective BRAF inhibitors is that they can often result in fast clinical responses, even within days, to treatment. This further emphasizes the importance of a fast assessment of BRAF mutation status in patients with advanced disease and acute deterioration.

In the current study the *BRAF* mutational status of retrospective clinical FFPE tissue sections was tested with Idylla™ in a routine clinical setting at six different centers. The results were compared to results obtained before with routine reference methods, which had been used to guide anticancer treatment. The overall concordance between Idylla™ and the confirmed test results in malignant melanoma was found to be 97.84%, and increases to 99.28% when taking into account that identifying the presence of a V600 mutation, irrespective of its exact nature, is sufficient to start BRAF-targeted therapy. These values confirm the high concordance values observed before when analyzing up to 31 years old FFPE tumor samples of a variety of human malignancies (Janku et al., 2015; Schiefer et al., 2015).

Apart from detecting V600E, Idylla™ also enables reliable detection of V600D, V600K, V600R, and V600M. The V600K mutation constitutes 19% of the BRAF mutations in metastatic melanoma patients (Menzies et al., 2012). Patients with this mutation are eligible to vemurafenib and dabrafenib therapy but some routine BRAF tests do not detect all non-V600E mutations, e.g., the cobas® 4800 BRAF V600 Mutation Test detects only 70% of V600K (Anderson et al., 2012). According to the manufacturer's instructions accompanying the cobas® 4800 BRAF V600 Mutation Test, a mutation was reported by cross-reactivity when V600D was present in more than 10% of the sampled DNA, V600K when present in more than 35%, and V600E2 when present in more than 65% of the sampled DNA. Therefore some patients that might benefit do not receive BRAF inhibitors when the current routine diagnostic tests are used, but would do if Idylla™ would be used to assess *BRAF* mutational status. Moreover, Idylla™ is also able to detect V600D, V600R, and V600M mutations that are implicated in the response to BRAF inhibitors as well (Klein et al., 2013; Parakh et al., 2015; Chapman et al., 2011; McArthur et al., 2014; Falchook et al., 2012; Yang et al., 2010; Ponti et al., 2012). In the current study, Idylla™ enabled detection of a V600K/R/M mutation while the reference method did not, in 6 samples.

Given the need for molecular pathology infrastructure and specialized staff, the routine reference methods are often performed by a third party. This results in longer turnaround times from sample to test result (days or weeks instead of hours), which could delay therapeutic decisions. In particular in the case of rapidly progressing diseases like advanced melanoma, such delays may negatively affect patient care, as a fast assessment of *BRAF* mutation status can be of critical importance. As Idylla™ can be easily implemented in any pathology laboratory setting and as the turnaround time to obtain test results is very short, the incorporation of the *BRAF* mutational status in the initial histological report is possible and targeted therapy can readily be initiated.

According to the Instructions for Use, Idylla™ requires a minimal tissue area of the specimen of 50 mm^2 when 5 μm FFPE tissue sections are used and of 25 mm^2 when using 10 μm FFPE tissue sections. The minimal tumor cell percentage in samples for Idylla™ is 50% to guarantee reliable or valid results. In case this lower limit is not met, macrodissection should be performed. However in the current study, for most of the samples not meeting these requirements Idylla™ was still able to determine *BRAF* mutational status. It is noteworthy that Idylla™ also implies upper limits to tissue area for correct operation, i.e., 600 mm^2 when 5 μm FFPE tissue sections are used and 300 mm^2 for 10 μm FFPE tissue sections; the importance of this upper limit was illustrated with Sample 3, for which an insufficient DNA input call was obtained.

Idylla™ has a sensitivity of 1% versus the 5% sensitivity for most PCR-based methods, including cobas® 4800 BRAF V600 Mutation Test and versus 5–10% for Next Generation Sequencing methods. In 7 instances, no mutation was detected by the routine reference method but Idylla™ identified a V600E/E2/D or V600K/R/M mutation whose presence was confirmed when a third method was used; this might be due to the higher sensitivity, the capability to identify more mutations, and the standardized workflow of Idylla™.

As Idylla™ Mutation Tests are always designed to qualitatively detect specific mutations, e.g., V600E/E2/D or V600K/R/M, they are not intended to determine all mutations within the exon or to quantify allelic frequency. Other more specialized molecular techniques should be utilized when complete overviews of all mutations present or when quantitative information would be needed. In general, the Idylla™ System was perceived to be very user-friendly, with very little hands-on time and short sample-to-result times being major advantages. The workflow (including deparaffinization, liberation of DNA, real-time PCR amplification, and target detection) is automated, and due to the set-up the risk of cross-contamination is minimal. At the down-side it was mentioned that DNA (or lysate) cannot be withdrawn from the cartridge and stored for future use.

In conclusion, the Idylla™ BRAF Mutation Test on the Idylla™ platform enables the fully integrated and automated testing of BRAF V600 mutational status directly on FFPE tumor tissue with increased sensitivity, ease-of-use, and much shorter turnaround time compared to existing diagnostic tests. Consequently, Idylla™ is an excellent tool for the rapid, simple and highly reliable analysis of therapeutically relevant

BRAF mutations in malignant melanoma, in particular for diagnostic units without or with limited molecular expertise and infrastructure.

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