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Rapid *BRAF* mutation tests in patients with advanced melanoma: comparison of immunohistochemistry, Droplet Digital PCR, and the Idylla Mutation Platform

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***BRAF* mutational testing has become a common practice in the diagnostic process of patients with advanced melanoma. Although time-consuming, DNA sequencing techniques are the current gold standard for mutational testing. However, in certain clinical situations, a rapid test result is required. In this study, the performance of three rapid BRAF mutation tests was compared. Thirty-nine formalin-fixed paraffin-embedded melanoma tissue samples collected between 2007 and 2014 at a single center were included. These samples were analyzed by immunohistochemistry using the anti-BRAF-V600E (VE1) mouse monocolonal antibody (BRAF-VE1 IHC), a V600E-specific Droplet Digital PCR Test, and the Idylla *BRAF*-Mutation Test (Idylla). Results were compared with the results of conventional *BRAF* mutation testing, performed using high-resolution melting analysis followed by Sanger sequencing. Next-generation sequencing was performed on samples with discordant results. The Idylla test and Droplet Digital PCR Test correctly identified all mutated and wild-type samples. BRAF-VE1 IHC showed one discordant result. The Idylla test could identify *BRAF*-V600 mutations other than *BRAF*-V600E and was the fastest and least laborious test. The Idylla Mutation Test is the most suitable test for rapid BRAF testing in clinical situations on the basis of the broad coverage of treatment-responsive mutations and the fast procedure without the need to perform a DNA isolation step. *Melanoma Res* 28:96–104 Copyright © 2018 The Author(s). Published by Wolters Kluwer Health, Inc.**

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Introduction

Single-point mutations in the gene encoding *BRAF* function as an oncogenic driver of cutaneous melanoma. These mutations occur in 40–60% of all cutaneous melanomas [1,2]. BRAF is a member of the kinase family of RAF kinases. This serine/threonine kinase acts as a signaling protein in the mitogen-activated protein kinase (MAPK) pathway, which regulates cell growth, survival, and differentiation [3]. A somatic mutation affecting the valine residue at position 600 results in a mutated hyperactive BRAF protein that induces constitutive signaling through the MAPK pathway and enables oncogenesis. The most common mutation, detected in around 75% of all *BRAF* mutation-positive melanoma, is a substitution of glutamic acid for valine at codon 600, BRAF p. (V600E) [1]. Unraveling the crystal structure of the mutated BRAF protein [4] led to the development of several small-molecule BRAF-specific inhibitors. Vemurafenib and dabrafenib have been approved for the treatment of *BRAF* mutant metastatic melanoma both in North America and in Europe [5,6]. These inhibitors have led to improvement in progression-free as well as overall survival compared with standard treatment. Besides the *BRAF*-V600E mutation, several other *BRAF* mutations have been detected in melanoma with variable responsiveness to treatment with BRAF inhibitors [7]. The most frequently detected non-V600E mutation is *BRAF*-V600K, a substitution of valine to leucine at codon 600, accounting for 15–20% of *BRAF* mutations in melanoma [8]. *BRAF*-V600K mutant melanomas are also responsive to BRAF inhibitor therapy, although to a lesser extent than *BRAF*-V600E [9]. Other rare mutations such as *BRAF*-V600D/R/M occur in 1–2% of patients [7]. Evidence of treatment responses to BRAF inhibitors in

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melanoma harboring these rare V600 mutations has been provided by case reports and preclinical studies [10,11].

Molecular diagnostic testing of the BRAF-V600 and other relevant predictive biomarkers is becoming routine practice in treatment decision-making. Mutation detection is performed routinely on pretreatment tumor biopsies or resection specimens. According to national and international guidelines, mutational testing is mandatory in advanced-stage melanoma (stage IIIC or IV) and before the initiation of systemic treatment [12,13]. Mutational testing should, at least, include all known activating *BRAF* mutations. For the detection of mutations, a variety of techniques are used including high-resolution melting (HRM) analysis, followed by sequencing, Sanger bidirectional sequencing, pyrosequencing, and, recently becoming more common, next-generation sequencing (NGS) using dedicated gene-panels [14]. These techniques are often expensive, labor-intensive, and time-consuming. In addition, they depend on sufficient amounts of DNA (10–500 ng) and a certain percentage of neoplastic cells (>5–20%) to be able to detect clinically relevant mutations. A problem arises when no representative biopsy is available for mutational testing. In addition, in patients with rapidly progressive melanoma and high morbidity, there is a need for a test with a shorter turnaround time, especially because in BRAF mutated patients, responses and clinical improvement can be observed within several days after the start of BRAF-targeted therapy. In recent years, several such molecular tests have become available. The first test was the Cobas 4800 BRAF-V600 mutation test, which was developed as a companion diagnostic test for use in the clinical trials with vemurafenib and was used widely after vemurafenib was approved as a treatment for advanced melanoma [15]. This Cobas test is a real-time PCR test that showed higher sensitivity and specificity than direct bidirectional sequencing. Thereafter, other *BRAF*-V600 mutation-specific tests were developed and reported. For instance, Droplet Digital PCR (ddPCR) assays to detect *BRAF*-V600E mutations showed high concordance with pyrosequencing and HRM analysis tests [16]. Quantitative ddPCR assays have a high analytical sensitivity that enables accurate screening of *BRAF*-V600E mutations in tissues with low numbers of neoplastic cells [17]. Furthermore, immunohistochemistry (IHC) using a BRAF-V600E-specific monoclonal antibody may also be used as a rapid test for the detection of BRAF-V600E-mutated protein [18,19]. More recently, the Idylla *BRAF* Mutation Test, a rapid and fully automated test performing both DNA extraction from formalin-fixed paraffin-embedded (FFPE) slides and real-time PCR, showed highly concordant results compared with conventional molecular tests [20–22].

In this study, the performance of three different rapid BRAF mutation tests was compared. The results of IHC with the BRAF-VE1 antibody, *BRAF*-V600E mutation ddPCR test, and the Idylla *BRAF* Mutation Test were compared with the conventional *BRAF* mutation test using HRM/sequencing. In addition, we compared the three tests with respect to several other aspects such as turnaround times and costs.

Materials and methods

Samples

A cohort of FFPE melanoma tissue samples from 39 patients with a known *BRAF*-V600 mutation status was selected for this study. BRAF mutation status was determined in routine clinical practice using HRM for all samples, with subsequent Sanger sequencing of HRM-positive samples. All tissue samples were collected between 2007 and 2014 and stored in the pathology archives of our center. Samples could be derived from excision of primary melanoma, lymph node dissection, biopsies of intestinal metastases, or resection of other hematogenous metastases. We selected 39 FFPE samples randomly. Efforts were made to include an equal number of *BRAF* mutation-positive and *BRAF*-negative samples. In addition to 18 *BRAF*-V600E-positive samples, two *BRAF*-V600K-positive and one *BRAF*-V600R-positive sample were included. Before the tests were performed, an experienced pathologist evaluated the tumor content of tissue samples by estimating the percentage of neoplastic cells on hematoxylin and eosin-stained whole slides. The percentage of neoplastic cells in the samples ranged from 2 to 95%. For HRM and ddPCR, macrodissection was used to enrich the percentage of neoplastic cells in the samples. All procedures and protocols were performed according to the guidelines for good clinical practice. HRM and Sanger sequencing tests were performed as part of the routine diagnostic approach and the outcome of these tests was documented in the patient file and communicated with the medical oncologists and patients. This is a retrospective clinical validation study; therefore, no consent was required from the Internal Review Board to analyze clinical patient data under the Dutch Law for human medical research (WMO). Data were encoded so that they were not traceable to the individual patient, according to national ethical guidelines ('Code for Proper Secondary Use of Human Tissue', Dutch Federation of Medical Scientific Societies).

Study design

In this study, three BRAF-V600 mutation tests were compared: IHC with the BRAF-VE1 monoclonal antibody, ddPCR, and the Idylla *BRAF* Mutation Test (Biocartis, Mechelen, Belgium). The performance of these three tests was compared with HRM/Sanger sequencing as the gold standard. In addition, turnaround time, hands-on time, costs, limit of detection, failure rate, detectable BRAF mutations, CE-IVD marking, and the amount of FFPE material required for the test were determined. Discordant results were tested using NGS.

All molecular tests were performed in the CCKL/ISO15189-Accredited Laboratory of Molecular Pathology at the University Medical Center Groningen. All standard precautions were taken to avoid contamination of amplification products using separate laboratories for pre-PCR and post-PCR handling. To avoid cross-contamination, a new microtome blade was used each time a new sample was sectioned.

DNA isolation for high-resolution melting analysis/sequencing and Droplet Digital PCR

Four 10 μm slices were cut from FFPE tissue blocks for DNA isolation. Tumor cell-rich areas marked by an experienced pathologist were scraped from the slides using a scalpel. Subsequently, DNA was extracted using either the Cobas DNA Sample Preparation Kit (Roche, Basel, Switzerland) or using the Proteinase K DNA isolation (Roche, Pleasanton, California, USA) method as described previously [23]. The concentration of DNA was determined on the NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) for HRM/sequencing and on a Qubit Fluorometer (Life Technologies, Carlsbad, California, USA) for ddPCR or both.

High-resolution melting analysis and Sanger DNA sequencing

In all selected melanoma samples, the *BRAF* mutation status was determined by HRM/Sanger sequencing. For the detection of mutations in exon 15 of the *BRAF* gene, 100 ng genomic DNA was analyzed by PCR using specific primers covering exon 15 (NM_004333), followed by direct bidirectional Sanger sequencing as reported previously for the detection of mutations in the EGFR and KRAS gene [23]. Briefly, the PCR for the HRM analysis was performed on a LightCycler 480 (Roche, Basel, Switzerland). PCR reaction mixtures with a final volume of 20 μl contained 500 nmol/l forward primer (BRAF1F: 5′-CCT AAA CTC TTC ATA ATG CTT GCT C-3′), 500 nmol/l reverse primer (BRAF1R: 5′-CCA CAA AAT GGA TCC AGA CA-3′) [all primers were purchased from IDT (Leuven, Belgium), and 10 ng DNA in 1× HRM Mastermix from Roche]. The cycling and melting conditions were as follows: one cycle of 95°C/5 min; 50 cycles of 95°C/30 s; 65–54°C/30 s at 0.06°C/s; 72°C/30 s; and one cycle of 72°C/60 s, 95°C/20 s, 55°C/20 s with a final melting step: 75–99°C at 0.06°C/s and continuous recording of the fluorescent level. The change in fluorescence is converted into a melting peak by plotting the negative derivative of the fluorescent signal corresponding to the temperature ($-dF/dT$) on the LightCycler 480 software.

The original genomic DNA of cases with an abnormal HRM melting curve, characteristic for the presence of a mutation, was subjected to a direct bidirectional Sanger sequence analysis to identify the specific *BRAF* mutation as described for EGFR mutation detection [23] using BRAF-specific sequence primers: BRAF2F: 5′-CAT AAT GCT TGC TCT GAT AGG AAA-3′ and BRAF2R2: 5′-TCA GCA GCA TCT CAG GGC CAA A-3′.

BRAF-VE1 immunohistochemistry

IHC staining of the BRAF-V600E mutant protein was performed using the anti-BRAF-V600E mouse monoclonal antibody, VE1 catalog number 790–4855 (Ventana Medical Systems Inc., Tucson, Arizona, USA). IHC was performed on a tissue microarray (TMA). The TMA was constructed from three small cores (0.6 mm) from the target FFPE tissue block that were subsequently embedded in a recipient master paraffin block as reported previously [24]. As a positive control, two *BRAF*-V600E-positive papillary thyroid tissue samples and as a negative control liver and tonsil tissue samples were added. Sections of 4 μm were cut and these sections were mounted on a glass slide and stained with the VE1 monoclonal antibody. Staining was performed on a BenchMark ULTRA stainer (Ventana). FFPE sections were pretreated with the Tris-based buffer CC1 (Ventana) and thereafter incubated with undiluted VE1 antibody at 36°C for 60 min. The staining results were scored from 0 to 3+ as reported previously by four observers independently without knowledge of mutation status [18]. Discordant results were discussed until a consensus was reached. A score of 1–3 was considered a positive staining [25].

BRAF-V600E mutation Droplet Digital PCR

The ddPCR reaction was performed using 1.8 ng genomic DNA according to the manufacturer's instruction (Bio-Rad, Hercules, California, USA). The reaction mixture consists of up to 66 ng of genomic DNA, 11 μl ddPCR Supermix for probes (no dUTP), and 1 μl BRAF-V600E (FAM probe) and BRAF-V600 wild-type (HEX probe) assay (Bio-Rad ddPCR assay BRAF_dHsa CP2000027 and BRAF_dHsaCP2000028) in a total volume of 22 μl (Bio-Rad). Twenty microlitres was transferred to the cartridge and after the addition of 70 μl Droplet Generation Oil (Bio-Rad), thousands of nano-sized droplets were generated using the Droplet Generator QX100. PCR was performed on a T100 Thermal Cycler (Bio-Rad) under the following cycling conditions: 10 min at 95°C, 40 cycles of 95°C for 30 s, 55°C for 1 min, followed by 98°C for 10 min (ramp rate 2.5°C/s). Samples were transferred to the QX200 Droplet Reader (Bio-Rad) for fluorescent measurement of FAM and HEX probes and data were analyzed using Quantasoft software, version 1.7.4 (Quantasoft, Prague, Czech Republic). Samples were defined as positive when three or more FAM/HEX-positive droplets were detected with no positive droplets in the template controls. The fractional abundance was based on the ratio between mutant and wild-type droplets after correction with the

Poisson distribution (calculated using the Quantasoft software). The limit of detection was determined by serial dilution of a positive control sample using 20 ng at 0.1%.

Idylla *BRAF* Mutation Test

The Idylla (Biocartis) is a fully automated real-time-PCR-based *BRAF* mutation test. For the Idylla test, fresh slides were cut and the percentage of neoplastic cells in the whole section was estimated. This diagnostic platform uses disposable cartridges in which 5–10 µm FFPE tissue sections were mounted without any preparation such as deparaffinization or enrichment for neoplastic cells. The test consists of three allele-specific PCR reactions that enable identification of *BRAF* wild-type, *BRAF*-V600E/E2/D, or *BRAF*-V600K/R/M sequences. The detection limit, according to the manufacturer, was set at the conditions of 25 mm^2 FFPE tissue present in a 5–10 µm slide and a neoplastic cell content of more than 50%. In contrast to these recommendations, we included all samples irrespective of neoplastic cells' content in this study and no enrichment of neoplastic cells by macrodissection was performed to minimize hands-on time.

Next-generation DNA sequencing for confirmation of mutation status

Specimens with discordant test results between HRM/Sanger and BRAF-VE1 IHC, *BRAF*-V600E ddPCR or Idylla testing were retested using an independent, quantitative NGS assay using an in-house hotspot panel including exon 15 of the *BRAF* (NM_004333) gene (version PGMv001) on the IonTorrent (Thermo Fisher Scientific Inc.) sequencing platform (*http://www.moloncopath.nl*). Genomic DNA of 10 ng from each sample containing at least 20% neoplastic cells was used to prepare barcoded libraries using IonXpress barcoded adapters (Thermo Fisher Scientific Inc.). Libraries were combined to a final concentration of 100 pmol using the Ion Library Quantification Kit (Thermo Fisher Scientific Inc.) and emulsion PCR was performed using the IonTorrent OneTouch TM2 system. Samples were sequenced on the IonTorrent semiconductor sequencer using Ion 316 or 318 chips. Sequencing reads were aligned based on the Human Genome, version 19 using Sequence Pilot v4.2.0 (JSI Medical Systems GmbH, Ettenheim, Germany). The cutoff was set at a mutant allele frequency of more than 5%.

Statistical analysis

Descriptive statistics were used to summarize the data. All parameters were presented as frequencies or percentages. Sensitivity, specificity, and positive and negative predictive values were calculated using HMR/Sanger sequencing as the reference. All statistical analyses were carried out using SPSS (released 2013, IBM SPSS Statistics for Windows, version 22.0; IBM Corp., Armonk, New York, USA).

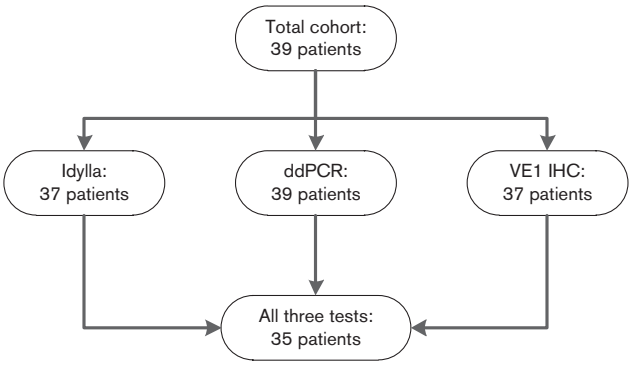
Results

FFPE tumor tissue blocks from 39 patients with a known *BRAF* exon 15 mutation status were included in this study (Supplementary Table 1, Supplemental digital content 1, *http://links.lww.com/MR/A22*). Because of the limited amount of tumor tissue or neoplastic cell percentage below the minimal cutoff in some of the tissue blocks, not all BRAF assays could be performed on all tissue samples (Fig. 1). First, a TMA was generated from the 37 samples with sufficient tumor tissue left in the FFPE blocks, IHC using the BRAF-VE1 antibody was performed on this TMA. DNA from all 39 patients was available for ddPCR. For 20 samples, DNA was available from previous mutation testing and for the remaining 19 samples, new DNA was extracted. For the Idylla test, fresh FFPE sections were cut from the 37 samples with sufficient remaining tissue. In 35 samples, the same tissue blocks were used for the Idylla test as were used for generating the TMA. In two patients (case nos 25 and 34), two tissue blocks from the same tumor sample were used to perform all three tests. These were considered the same tumor sample. All three tests were performed in 35 samples, allowing for interassay comparison (Fig. 1).

Results of the Ventana BRAF-VE1 immunohistochemistry

Two tumor samples from our selection did not contain sufficient neoplastic cells to be evaluated by IHC. The inter-observer agreement on IHC scoring between 4 independent observers was high. Discrepancies in scoring ($n = 2$) were resolved by consensus review with all observers together at multiheaded microscope. Of the 37 samples, 16 were scored as BRAF-VE1 positive and 21 as negative. One was difficult to interpret because of melanin pigment and was scored as negative by consensus review. In 35 of 37 samples, the BRAF-VE1 staining was in concordance with HRM/Sanger sequencing data (agreement of 95%) (Table 1). One of the discordant samples (case no. 4) showed a positive staining

Fig. 1



Flow chart showing the number of samples analyzed by the three different rapid *BRAF* mutation tests. ddPCR, Droplet Digital PCR.

Table 1 Results of the three rapid *BRAF* mutation test

	HRM/sequencing results				
Results of the rapid *BRAF* mutation tests	*BRAF*-V600E	*BRAF*-V600K	*BRAF*-V600R	BRAF WT	Total
BRAF-VE1 immunohistochemistry					
BRAF-V600E	15	0	0	1[a]	16
BRAF WT	1[c]	2	1	17	21
Total	16	2	1	18	37
BRAF-V600E ddPCR[b]					
BRAF-V600E	17	0	0	1[a]	18
BRAF WT	0	2	1	18	21
Total	17	2	1	19	39
Idylla BRAF Mutation Test[b]					
BRAF-V600E/V600E2/V600D	17	0	0	1[a]	18
BRAF-V600K/V600R/V600M	0	2	1	0	3
BRAF WT	0	0	0	16	16
Total	17	2	1	17	37

HRM and reflex sequencing results were considered the 'gold standard'.
ddPCR, Droplet Digital PCR; HRM, high-resolution melting analysis; PPV, positive predictive value; NPV, negative predictive value; WT, wildtype.
[a]Next-generation sequencing confirms the presence of the BRAF-V600E (c.1799T > A) mutation.
[b]Test results: sensitivity 100%, specificity 95% for ddPCR and 94% for Idylla, PPV 94% for ddPCR and 95% for Idylla, NPV 100%. Test results after discordant resolution by next-generation sequencing: sensitivity 100%, specificity 100%, PPV 100%, NPV 100%.
[c]True false-negative based on HRM/sequencing, Idylla, and ddPCR. Test results for BRAF-V600-VE1: sensitivity 94%, specificity 95%, PPV 94%, NPV 95%. Test results after discordant resolution by next-generation sequencing: sensitivity 94%, specificity 100%, PPV 100%, NPV 95%.

(consensus scoring: 2+), but was tested as *BRAF* wild type by HRM only (Supplementary Table 1, Supplemental digital content 1, *http://links.lww.com/MR/A22*). The second discordant sample (case no. 23) was BRAF-VE1 negative (consensus scoring: 0) despite the presence of a *BRAF*-V600E mutation and should be considered as a false negative for the IHC. In three cases with non-*BRAF*-V600E mutations (*BRAF*-V600K/R), BRAF-VE1 staining was truly negative. In summary, the BRAF-VE1 IHC showed a sensitivity of 94% and a specificity of 95% for *BRAF*-V600E-positive cases. The only discordant sample (case no. 4) was subjected to NGS analysis and indicated the presence of a *BRAF*-V600E (c.1799T > A) mutation in agreement with the BRAF-VE1 IHC result.

Results of the *BRAF*-V600E Droplet Digital PCR

In 38 of the 39 samples, the ddPCR results were in accordance with the *BRAF* mutation status (agreement of 97%) (Table 1). The only discordant sample (case no. 4) showed a positive ddPCR (fractional abundance of 18%), but was tested as *BRAF* wild type by HRM only (Supplementary Table 1, Supplemental digital content 1, *http://links.lww.com/MR/A22*). In all 18 samples without *BRAF* exon 15 mutations and three cases with the non-*BRAF*-V600E mutation (*BRAF*-V600K/R), *BRAF*-V600E ddPCR was negative. Thus, the ddPCR showed a sensitivity of 100% and a specificity of 95% for *BRAF*-V600E. The only discordant sample (case no. 4) was subjected to an NGS analysis and indicated the presence of a *BRAF*-V600E (c.1799T > A) mutation in agreement with the *BRAF*-V600-ddPCR result.

Results of the Idylla *BRAF* Mutation Test

Because of the absence of sufficient neoplastic melanoma cells in two FFPE blocks and no other available tissue sample, the Idylla test was performed on 37 of 39 samples (Fig. 1). All samples with sufficient tumor material left were analyzed irrespective of the neoplastic cell content. The percentage of neoplastic cells ranged from 2 to 95%. Twelve of the 37 samples had a percentage of neoplastic cells of less than 50% (Supplementary Table 1, Supplemental digital content 1, *http://links.lww.com/MR/A22*).

Results marked by the Idylla test as 'No mutation detected in *BRAF* codon 600' with an additional mark 'V600K/R/M-mutation less than 5% may not be detected' ($n = 7$, case nos 3, 13, 27, 31, 33, 34, 36) were repeated using two 10 µm tissue slides and the results were confirmed. Results marked with 'Invalid' (case no. 6) or 'insufficient DNA input' (case no. 33) were also reanalyzed using two 10 µm tissue slides. For case no. 6, the second run was now valid and showed a *BRAF* wild-type genotype. Case no. 33 also had insufficient DNA input in the second run. Re-evaluation by the pathologist showed insufficient tissue in this tissue block. The analysis of another tissue block from the same sample yielded a valid result, which was *BRAF* wild type. In all 16 samples without *BRAF* exon 15 mutations, the Idylla test was negative. In three cases with *BRAF*-V600K (case nos 5 and 9) and V600R (case no. 22) mutation, the Idylla test correctly identified these genotypes as V600K/V600R/V600M. Only one sample (case no. 4) showed a positive result (V600E/V600E2/V600D), but was tested as *BRAF* wild type by HRM.

In 36 of the 37 samples on which the Idylla test was performed, the results were in accordance with the results obtained with HRM/sequencing (agreement 97.3%) (Table 1). In summary, the Idylla *BRAF* Mutation Test showed a sensitivity of 100% and a specificity of 94% for *BRAF*-V600E/E2/D and *BRAF*-V600K/R/M. The only discordant sample (case no. 4) showed a *BRAF*-V600E (c.1799T > A) mutation using NGS in agreement with the Idylla result.

Head-to-head BRAF mutation assays' comparison

All three BRAF mutation tests showed a high accuracy compared with the routine *BRAF* diagnostic test with HRM/sequencing performed previously for diagnostic purposes. In 35 patients, all three rapid *BRAF* mutation assays were performed. Only one of these cases (case no. 23) showed discordance between the tests. IHC of this sample was scored BRAF-VE1 negative, but ddPCR

and the Idylla showed a *BRAF*-V600E mutation. HRM and Sanger sequencing performed in the past also detected a *BRAF*-V600E mutation in case no. 23. The IHC result of this sample was considered a false-negative result.

In another sample (case no. 4), all three tests detected a *BRAF*-V600E mutation, whereas HRM performed in the past tested the sample as *BRAF* wild type. NGS analysis indicated the presence of a *BRAF*-V600E (c.1799T > A) mutation in 9% of DNA in agreement with the results detected in all three tests. The HRM result was therefore considered false negative. As the presence of this mutation in case no. 4 should be considered true positive, after discordant resolution by NGS, both the ddPCR and the Idylla test showed a sensitivity of 100% and a specificity of 100% for *BRAF*-V600 mutation testing.

In addition to test performance, the BRAF mutation tests were also compared with respect to several test characteristics (summarized in Table 2). All three rapid *BRAF* mutation tests had a lower limit of detection than DNA sequencing techniques. The most sensitive test was ddPCR, with a limit of detection of 0.02% neoplastic cells. The turnaround time of the tests varied from 92 min for the Idylla test to ~8 h for ddPCR. These 1-day turnaround times are significantly shorter than a turnaround time of 2–3 days for HRM/sequencing and 3–5 days for NGS. Similarly, the hands-on time of the rapid *BRAF* mutation tests ranged from 2 to 120 min (not including tissue selection and sectioning) and was significantly shorter than the hands-on time of HRM/sequencing and NGS (4 and 6 h, respectively). The *BRAF* Idylla test showed the shortest hands-on time.

Discussion

Molecular diagnostic testing of the BRAF-V600 and other relevant predictive biomarkers in advanced-stage melanoma (stage IIIC or IV) is routine practice for treatment decision-making according to both national and international guidelines. The clinical demand for mutation detection in multiple genes from a single tumor sample requires molecular diagnostic laboratories to develop rapid, high-throughput, highly sensitive, accurate, and parallel testing. To meet this demand, many laboratories use NGS [26]. However, multigene sequencing is a time-consuming method. As a consequence, a problem arises in rapidly progressive melanoma patients and in patients who are hospitalized in centers without the possibility to perform advanced genetic tests. Even in patients with rapidly progressive metastatic disease, clinical response to BRAF inhibitors can be evident within 1 day. A rapid test that detects a treatment-responsive BRAF mutation can ensure correct initiation of BRAF-inhibitory treatment while preventing unnecessary costs and side-effects of treating wild-type patients. In this comparative study, three rapid BRAF mutations tests were evaluated with respect to different aspects of their use in routine clinical practice and were compared with conventional tests using HRM and Sanger sequencing performed in the past.

All BRAF mutation assays in this comparative study showed a high sensitivity and specificity for *BRAF*-V600E mutation detection, although IHC had a lower sensitivity than the PCR tests because of one false-negative result (Table 1). The sensitivity and specificity of the BRAF-V600E-specific VE1 monoclonal antibody was in accordance with previous studies [18,19]. Both false-negative (10%) as well as false-positive (5%) results were found with the VE1 antibody in one of these studies [19]. In another study, the agreement between the allele-specific TaqMan assay and IHC using the *BRAF*-VE1 antibody was high (89/97). The eight discordant cases all represented false-positive results and all showed only weak to moderate staining intensity [27]. In our study, IHC was performed on a TMA with 0.6 mm tissue samples and the false-negative result (case no. 23) could be the result of sampling error in a tumor with a heterogeneous expression of mutant BRAF. Both PCR tests

Table 2 Characteristics of BRAF mutation tests

Test characteristics	HRM/Sanger sequencing	Next-generation sequencing	Idylla	ddPCR	VE1 IHC
CE-IVD	No	No	Yes	No[a]	Yes
BRAF mutation detection	Whole exon 15	Whole exon 15	V600E/E2/D/K/R/M	c.1799T > A (V600E)	V600E
Limit of detection[b]	20%	10%	1%	< 0.02%	Few cells
Failure rate[c]	4%	5%	3%	3%	3%
Turnaround time[d]	2–3 days	3–5 days	92 min	~8 h	140 min
Hands-on time[e]	~4 h	~6 h	< 2 min	~2 h	~5 min
Amount of material used	100–250 ng	≥ 10 ng	5–10 μm section	≥ 2 ng	3–4 μm section
Costs[f]	€175	€275	€150–170	€45	€122

ddPCR, Droplet Digital PCR; HRM, high-resolution melting analysis.
[a]Test is clinical-validated ISO15189.
[b]Defined as the amount of mutant DNA copies in the background of wild-type DNA copies for all PCR-based tests and as the number of mutated cells in a field of view for immunohistochemistry.
[c]Based on the study results for the Idylla, ddPCR, and VE1 IHC, and based on routine practice results in our center for HRM/Sanger sequencing and next-generation sequencing.
[d]Defined as the time from the start to the result of the test. Does not include sample preparation, cutting slides, quality control by the pathologist, and reporting of results.
[e]Time of manual labor that is required to perform the test. Does not include selection of blocks, cutting of sections, and reporting of results.
[f]Cost are list prices and do not include overhead, salary, maintenance, equipment, quality control-testing, biostatistician, etc. considered to be similar for all five tests.

showed a 100% sensitivity and specificity for the detection of *BRAF* mutations, which is also in accordance with comparable studies [16,20–22,28].

Furthermore, all tests have a low detection limit. The ddPCR is reported to be the most sensitive assay with a detection limit of 0.02% mutant DNA in a background of wild-type DNA [16]. The ddPCR assay used in our study center was validated using an input of 20 ng DNA, which resulted in a detection limit of 0.1% mutant DNA. Another advantage of this quantitative ddPCR is the ability to quantify the percentage of mutated DNA copies. For the Idylla test, we could detect a *BRAF* mutation in samples with as low as 2% neoplastic cells. This is much lower than the condition of more than 50% of the tumor cell content in tissue samples that is set by the manufacturer and was used as a minimal input in previous studies [21,22,29]. One other study described similar results when using this test without macrodissection [20]. Also, BRAF-VE1 IHC has a low limit of detection and allows for the detection of *BRAF*-V600E-mutated cells at the single-cell level. However, interpretation of staining results can be complicated in tissue slides that have marked melanin pigmentation. Therefore, the use of a red-colored immunostaining or Giemsa counterstaining can be considered [30].

In contrast to the ddPCR and BRAF-VE1 IHC, the Idylla test can detect other mutations in codon 600 besides the V600E mutation. This is of clinical relevance because some melanomas harbor non-V600E mutations that can be targeted effectively by BRAF inhibitors, such as the BRAF-V600K mutation [31]. The V600E mutation is the most common BRAF mutation, identified in ~75% of *BRAF* mutated melanomas [7]. The second most common mutation is V600K, which constitutes ~20% of all *BRAF* mutations in melanoma patients [7,8]. The remaining 5% of *BRAF* mutations are mainly found in codon 600, such as V600E2, V600D, V600R, and V600M [7]. All the aforementioned mutations can be detected by the Idylla test, although the Idylla is not able to make a distinction between V600E, V600E2, and V600D or between V600K, V600R, and V600M. Although melanoma with any of these mutations could respond to BRAF inhibitors, this test characteristic makes the Idylla test unsuitable for use in the USA as the Food and Drug Administration restricts BRAF inhibitor treatment to advanced melanoma with either the BRAF-V600E or BRAF-V600K mutations. The Idylla cannot detect *BRAF* mutations outside codon 600, which precludes detection of some rare non-V600 BRAF mutations, such a K601E and L597Q, that could respond to treatment with trametinib, an inhibitor of MEK1/2, a signaling protein acting downstream of BRAF in the MAPK pathway [32]. Because of the small number of non-V600E *BRAF* mutations in this study, we could not discern the accuracy of the BRAF tests for detecting these rare mutations. However, previous independent studies on testing for *BRAF*-V600 mutations in melanoma FFPE tissue biopsies comparing sequencing with BRAF-VE1 IHC [18,33], *BRAF*-V600E ddPCR [16], and *BRAF*-V600 Idylla [20,22] yielded similar data with high agreement.

The Idylla test and BRAF-VE1 IHC were less laborious than ddPCR and HRM/Sanger sequencing, the last two requiring significantly more hands-on time for tissue preparation (Table 2). The Idylla was the most rapid test; it is a fully automated test and produces final results within 90 min. IHC using an anti-BRAF-V600E monoclonal antibody is also fully automated, with a relatively short turnaround time of 140 min including additional scoring of the staining by a pathologist. The ddPCR has a significantly longer time to result than the other rapid *BRAF* mutation tests used in this study. This is a consequence of the multistep process of this assay that demands manual labor at multiple times during this process.

The costs of all rapid BRAF tests are considered to be similar in general (Table 2). Although the costs associated with Idylla and BRAF-VE1 monoclonal antibody testing are relatively high compared with the ddPCR, the Idylla and IHC tests require less hands-on time and therefore have less costs associated with human resources. For an objective comparison of the costs of these different tests, variable costs associated with maintenance, equipment, quality control, and overheads were not included in the equation because these are difficult to determine as these will vary between different countries and laboratories. As ddPCR and IHC testing only identifies BRAF-V600E mutations, only 75% of clinical-relevant BRAF-V600 mutations are detected. Rapid testing for relevant mutations other than the BRAF-V600E mutation would require additional testing with accordingly higher costs.

In all melanoma samples included in this study, HRM/Sanger sequencing was performed as a *BRAF* mutation test for diagnostic purposes. The results of the three rapid BRAF assays were in accordance with the HRM/sequencing results, except for a single sample (case no. 4). This sample was tested as *BRAF* wild type by HRM and was tested as BRAF-V600E by all other *BRAF* mutation assays in this study. The presence of a *BRAF*-V600E mutation was confirmed by independent NGS testing. This case showed that HRM has a lower sensitivity for *BRAF* detection than the other BRAF assays, which is consistent with the results of previous studies that compared HRM with other mutation tests [14,28]. In addition, as a condition for HRM, tumor samples should contain more than 50% neoplastic cells, which is much higher than the lowest content of 2% of neoplastic cells in which the Idylla could detect a *BRAF* mutation in this study. Furthermore, to achieve a tumor content of more than 50% neoplastic cells, macrodissection often has to be performed, which increases the risk of contamination. Currently, HRM/Sanger sequencing has been replaced

widely by NGS approaches. With a higher sensitivity for mutation detection, NGS can provide a broader molecular profile of an individual tumor for appropriate treatment decision-making, which is likely to become more important in the near future when additional molecular targets for the treatment of melanoma become available. Therefore, we want to emphasize that the rapid *BRAF* tests cannot replace NGS, and should only be performed in certain clinical situations that demand a rapid BRAF mutation analysis. When rapid BRAF tests are applied, it should always be complemented by NGS later on.

Besides melanoma, relatively high rates of activating BRAF mutations are encountered in colorectal cancer, thyroid cancer, and ovarian cancer [3]. Clinical trials with vemurafenib and dabrafenib have shown variable efficacy in these tumor types [34,35]. If BRAF inhibitor therapy receives regulatory approval for other tumor types in the future, rapid BRAF mutation tests will become relevant in these tumor types.

In the context of advanced melanoma, there is currently a clinical need to rapidly detect BRAF mutations. In this head-to-head comparison, the Idylla real-time PCR *BRAF* mutation test was found to be the most suitable test for rapid BRAF mutation detection. This test is fast and simple to perform and can therefore be implemented widely in any CCKL/ISO15189-accredited center with a molecular diagnostics department.

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Conflicts of interest

E.S. performed lectures for Illumina, Novartis, Pfizer, BioCartis, and is consultant in advisory boards for AstraZeneca, Pfizer, Novartis, and BioCartis (all fees to the Institution). For the remaining authors there are no conflicts of interest.

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