Special Report: Companion Diagnostics—Example of \textit{BRAF} Gene Mutation Testing to Select Patients with Melanoma for Treatment with \textit{BRAF} Kinase Inhibitors

Executive Summary

Background

Drug discovery and development generally follows one of two different paradigms: physiology-based discovery or target-based discovery. Physiologic-based drug discovery screens a large number of compounds using a physiologic readout such as an animal model or a cellular assay. Target-based drug discovery begins by identifying the function of a possible therapeutic target, such as an enzyme or a cell surface receptor, and its role in disease. Particularly in the latter case, patients often must be selected for treatment using a laboratory test (“companion diagnostic”) that identifies which patients have the target.

Examples of target-based discovery include imatinib, originally designed to inhibit the constitutively activated kinase product of the abnormal fusion gene \textit{BCR-ABL} found in chronic myelogenous leukemia (CML); and trastuzumab, an antibody designed to bind to HER2 receptors overexpressed on the cell surface of some types of breast cancer. In the case of imatinib, however, as nearly all patients with CML harbor the \textit{BCR-ABL} gene, a companion test to initially select patients for treatment is largely unnecessary. The HER2 companion test initially commercialized was not used in the clinical trials of trastuzumab and the subjectivity component of test readout was not initially appreciated, resulting in the design of improved HER2 tests and extended analyses of the quality and utility of HER2 companion testing. In some cases, companion test development and evaluation is retrospective, as in the case of KRAS mutation testing to select colorectal cancer patients likely to respond to cetuximab or panitumumab treatment. Thus, these are not the best examples of treatment-test co-development.

Recent examples of targeted drug design and companion test co-development (Table) include crizotinib (Pfizer, Inc.), which targets an abnormal fusion gene product in some patients with non-small cell lung cancer, and vemurafenib (Hoffmann-La Roche Ltd.) and dabrafenib (GlaxoSmithKline plc), both \textit{BRAF} inhibitors that target a mutated form of the \textit{BRAF} kinase, derived from the mutated \textit{BRAF} gene (\textit{BRAF}^{V600E}) occurring in 40–60% of melanoma tumors. This mutation results in a change from valine to glutamic acid at amino acid position 600 in the \textit{BRAF} protein. As these products were in development, the U.S. Food and Drug Administration (FDA) was developing a draft guidance for in vitro companion diagnostics, the major principles of which were likely applied to products nearing FDA submission, including crizotinib and vemurafenib. This guidance is important not only for its applicability to targeted drug development, but also for its impact on a broader category of “personalized” treatments that use a companion test to select patients most likely to respond, or to avoid treating patients likely to have serious adverse reactions.
Objective
An important question is whether the co-development process ensures sufficient validation of the companion diagnostic test, such that an independent evaluation becomes unnecessary. This Report will address that question using a targeted drug discovery example: the recently approved vemurafenib for certain patients with melanoma and its co-approved companion diagnostic test.

Search Strategy
The MEDLINE® database was searched (via PubMed) for articles using the terms “PLX4032,” “vemurafenib,” “V600E,” and “BRAF inhibitor,” all coupled with the term “melanoma.” The reference lists of relevant study publications and review articles were also examined. The meeting abstracts for the 2011 annual meeting of the American Society of Clinical Oncology were searched using the MEDLINE® search terms. If available, virtual presentations and slides were reviewed for key abstracts. The “grey literature” was consulted in the form of drug and laboratory test approval information released by the FDA, ongoing clinical trials from www.clinicaltrials.gov, and online searches for status and ancillary information.

Selection Criteria
For primary clinical data, we looked for trials of vemurafenib that used the final version of the companion test to select patients. Trials that used a prototype assay were also considered.

Main Results
The cobas® 4800 BRAF V600 Mutation Test real-time polymerase chain reaction (PCR) test is intended to detect the $BRAF^{V600E}$ mutation in formalin-fixed, paraffin-embedded (FFPE) tumor specimens from patients with advanced or metastatic melanoma and return a positive (mutation detected) or negative (mutation not detected) result. Once the test was co-approved with vemurafenib, extensive and detailed evidence regarding the analytic validity (technical performance) of the assay was available in the FDA Summary of Safety and Effectiveness and from the kit insert. Analytic validation data support a sensitive and robust assay for the detection of the V600E mutation in FFPE melanoma specimens.

One limitation was lack of complete data for calculating sensitivity and specificity. Sanger sequencing was used as a gold standard for FDA submission studies, but due to insensitivity at lower percentages of mutated alleles is an inappropriate standard. For the Phase III trial, samples from patients screened for the trial were sequenced, including those which were $BRAF^{WT}$; for the Phase II trial, only enrolled patients were sequenced, thus only percent agreement between the two methods could be calculated. Subsequent studies indicated a high percent agreement between

FISH: fluorescence in situ hybridization; PCR: polymerase chain reaction

<table>
<thead>
<tr>
<th>Drug</th>
<th>Indication</th>
<th>Molecular Indicator of Response/No Response</th>
<th>Companion Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imatinib</td>
<td>Chronic myelogenous leukemia (other indications not listed here)</td>
<td>$BCR-ABL$ kinase</td>
<td>Qualitative detection of $BCR-ABL$ fusion transcript</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>Breast cancer</td>
<td>HER2</td>
<td>Protein staining by immunohistochemistry; Gene amplification by FISH</td>
</tr>
<tr>
<td>Cetuximab, Panitumumab</td>
<td>Advanced colorectal cancer (other indications not listed here)</td>
<td>$KRAS$</td>
<td>Mutation detection, various methods</td>
</tr>
<tr>
<td>Crizotinib</td>
<td>Advanced non-small cell lung cancer</td>
<td>ALK fusion proteins</td>
<td>ALK gene rearrangements by FISH</td>
</tr>
<tr>
<td>Vemurafenib</td>
<td>Advanced melanoma</td>
<td>$BRAF$ V600 mutation</td>
<td>Real-time PCR $BRAF$ mutation detection</td>
</tr>
</tbody>
</table>

Table. Examples of Drugs Requiring Companion Diagnostic Tests
the cobas 4800 BRAF V600 Mutation Test and more sensitive sequencing methods, but insufficient information was available to allow recalculation of the Phase III sample data. Comparison with sequencing showed that in addition to very high percent agreement for the V600E mutation, the companion test also identified some, but not all, V600 variant mutations (e.g., V600K) as positive. Tumors with V600 variant mutations have a different amino acid substitution in the BRAF kinase.

When a therapeutic treatment is developed for a specific biological target that characterizes only some patients with a particular disease, and a test is co-developed to identify diseased patients with that target, studies supporting clinical validity (defined as the ability of the test to predict the disorder or phenotype of interest) and clinical utility (evidence of improved measurable clinical outcomes with test use) are no longer separate and sequential. Rather, the clinical studies of treatment benefit, which use the test to select patients for enrollment, provide evidence of both clinical validity and clinical utility. The primary evidence of clinical validity and utility for the cobas 4800 BRAF V600 Mutation Test is provided by the Phase III clinical trial of vemurafenib, which also supported the FDA approval of the drug. In addition, evidence from Phase I and Phase II trials was reviewed. All trials used enrichment trial designs, in which patient enrollment was based on a positive result for a V600 mutation in a tumor specimen.

The Phase I single-arm clinical trial of vemurafenib used a prototype assay to detect $\text{BRAF}^{\text{V600E}}$ mutations in enrolled patients. After dose determination, the extension phase of the study resulted in 81% of 52 patients responding according to Response Evaluation Criteria in Solid Tumors (RECIST); nearly all were partial responses.

The Phase II single-arm clinical trial is currently ongoing; interim results presented at a meeting showed a 55% objective response rate, median progression-free survival of 6.7 months, and median overall survival not reached at the time of analysis. Patients were selected for enrollment based on a finalized version of the cobas 4800 BRAF V600 Mutation Test.

The Phase III comparative trial of vemurafenib versus standard chemotherapy (dacarbazine) also enrolled patients based on the results of the finalized companion test. At a planned interim analysis, the results met the specified criteria for primary endpoints, and patients in the dacarbazine treatment arm were allowed to cross over to vemurafenib. At this time, median survival had not been reached; the hazard ratio for death was 0.37 (95% CI: 0.26–0.55). At 6 months, overall survival was 84% (95% CI: 78 to 89) for vemurafenib-treated patients and 64% (95% CI: 56 to 73) for dacarbazine-treated patients. Progression-free survival was evaluable in 549 patients; the hazard ratio for tumor progression was 0.26 (95% CI: 0.20–0.35). The median progression-free survival was estimated to be 5.3 months for patients treated with vemurafenib and 1.6 months for patients treated with dacarbazine. Tumor response was evaluable in 459 patients; the objective response rate was 48% in patients treated with vemurafenib versus 5% in those treated with dacarbazine. Only 2 patients treated with vemurafenib had a complete response.

A very small number of patients with V600 tumor mutations other than V600E were included in the Phase II and III clinical trials. No subgroup analyses were performed, although it was noted that some of these patients had at least partial responses.

**Author’s Comments and Conclusions**

The targeted drug-companion diagnostic test co-development process is a subset of a broader category of “personalized” treatments that use a companion test to select patients most likely to respond, or to avoid treating patients likely to have serious adverse reactions. For example, an evaluation of HLA-B*5701 testing as a companion test for abacavir resulted in the exclusion of test-positive patients from abacavir treatment and the elimination of immunologically confirmed, serious hypersensitivity reactions. In this case as in other examples, the companion test was developed and evaluated after drug approval. Going forward, as the molecular mechanisms and genetic influences of treatment actions are increasingly understood, companion tests are more likely to be developed in tandem. Targeted drug development, which depends on such an understanding, offers early examples of this process. In recognition, the FDA has provided draft guidance for a parallel and coordinated review and approval process in which both therapeutic
treatment and diagnostic test must be approvable, or neither will be approved. As justification, the FDA notes that when use of the treatment depends on the results of the companion test, then there are important concerns regarding the safety and effectiveness of both the therapeutic treatment and the companion diagnostic test.

This Report examined the data supporting the co-review and approval process using vemurafenib and its companion test, the cobas 4800 BRAF V600 Mutation Test, for the treatment of advanced/metastatic melanoma. Based on this example, general caveats to the co-development process require discussion. First, the vemurafenib Phase II and III clinical studies were all of enrichment design, enrolling patients based on a positive result from the companion test. The feasibility of this trial design relies on the knowledge from targeted drug design and from preclinical studies that melanoma tumors with a V600 mutation are likely to respond to vemurafenib, whereas tumors without a V600 mutation are unlikely to respond. As a result, smaller, more efficient trials can be conducted to determine drug efficacy. The trade-off, however, is the loss of information regarding the effect of vemurafenib on V600 wild-type melanoma tumors. Although preclinical data suggested that vemurafenib may potentiate tumor growth in V600 wild-type tumors, the comparable effect in patients remains unknown. Thus, the co-development process results in a narrow indication for treatment.

A limitation of the vemurafenib clinical studies was the absence of data on tumor tissue heterogeneity or homogeneity for the \textit{BRAF}^{V600E} mutation. The cobas 4800 BRAF V600 Mutation Test provides qualitative mutation detection using extracted DNA from a melanoma tissue specimen. Although it is highly sensitive for samples with low percent mutation in studies of analytic validity, clinical trials did not evaluate percent mutation within tumors, i.e., tumor heterogeneity, and correlate that result with response/nonresponse, or degree of response. Studies published by other groups suggest that \textit{BRAF} mutations are early events in the development of melanocytic tumors, but alone are insufficient to cause primary tumor formation and progression. Tissue from primary tumors positive for \textit{BRAF} mutations appears to be heterogeneous at a cellular level, but related metastases are consistently \textit{BRAF} positive if the primary tested positive. Immunohistochemistry studies suggest that metastases are homogenous in terms of mutated protein reactivity, but intensity of staining can be variable, and a more quantitative correlation of \textit{BRAF} mutation with treatment outcome may be useful. Although this information is preliminary, it is conceivable that improved companion tests or tests that enhance the information provided by the basic companion test may be developed; whether the co-development review process allows for such development is unclear.

In recent years, molecular tests have been carefully evaluated by groups like the EGAPP Working Group\(^1\) and the Agency for Healthcare Research and Quality's various evidence-based assessment programs.\(^2\) This is because FDA review of diagnostic tests does not usually encompass clinical utility and, in fact, most molecular tests are laboratory-developed tests not submitted to FDA for review.

The companion test development process results in the selection of patient groups that are either highly likely or highly unlikely to respond, seemingly a special case of a predictive test. And while targeted drug development does represent a new era of structural drug design that is more likely to elicit strong and predictable responses, general considerations of test evaluation are not unique. In general, predictive tests attempt to separate a patient population into those with a higher versus a lower likelihood of response to a treatment. Often the separation of groups is insufficient for clinical utility for individual patients, although separation of groups may be statistically significant (clinical validity). Targeted drugs increase the likelihood of response such that clinical utility is more often shown.

\(^1\) Evaluation of Genomic Applications in Practice and Prevention, information available at www.egappreviews.org.
\(^2\) For example, Effective Health Care and Evidence-based Practice programs; U.S. Preventive Services Task Force; see www.ahrq.gov.
This Report specifically asked whether the companion diagnostic co-review and approval process generated sufficient evidence of clinical utility in the example reviewed, such that additional evaluation would not be necessary for future companion tests approved via this process.

The cobas 4800 BRAF V600 Mutation Test was developed sufficiently early in the overall drug-test validation process such that a finalized version of the test was used in both Phase II and key Phase III clinical studies, the latter of which resulted in the clinical data submitted to the FDA. The companion test was co-submitted as a Class III device, requiring a higher level of support data. This was likely required because of potentially higher risk to the patient based on test results than for other types of laboratory tests that are classified as Class I or II devices. Extensive data support analytic validity, the technical performance of the test.

The results of the Phase III trial, supported by the results of the earlier trials, support the clinical validity and clinical utility of the cobas 4800 BRAF V600 Mutation Test, the companion diagnostic test for vemurafenib. Using the test to select patients for treatment results in improved outcomes compared to the usual standard of care, dacarbazine. In addition, comparison of these results with the trial results of the recently approved ipilimumab, suggests that treatment with vemurafenib results in improved outcomes compared to ipilimumab. Ipilimumab is notable as the first therapy to show a survival advantage in a Phase III trial for patients with advanced melanoma, and while vemurafenib was in clinical trials, may have become the new treatment standard for late stage disease and thus is an important comparator. Currently, a trial is underway testing the combination of vemurafenib and ipilimumab (ClinicalTrials.gov Identifier NCT01400451).

Some important limitations and gaps in knowledge were identified:

- As noted, the vemurafenib companion test identifies some melanoma tumors carrying \textit{BRAF} V600 mutations other than V600E as positive; follow-up on vemurafenib response in this patient population will be important.
- Use of commercially available, non-FDA-approved laboratory developed tests for the \textit{BRAF}^{V600E} mutations to select patients for vemurafenib treatment is not straightforwardly addressed by the FDA guidance on in vitro companion diagnostics or by other in vitro diagnostic or clinical laboratory regulations. It is possible that several different tests could be used for this purpose; impact on treatment outcomes is unknown.
- Knowledge of the impact of tumor heterogeneity with respect to the \textit{BRAF} mutation on vemurafenib treatment outcomes may inform patient selection.
- In general, it should be remembered that the development process, using enrichment trial designs, is an efficient process that provides information regarding patients with the molecular target, but provides no information regarding all other patients, who may have received all other treatment possibilities, and for whom any new available treatment may be attractive.

We conclude that the FDA process for coordinated review and co-approval of both therapeutic treatment and associated companion diagnostic test ensured sufficient validation of the companion diagnostic in the case of vemurafenib and its companion test, the cobas 4800 BRAF V600 Mutation Test. Important limitations and knowledge gaps specific to the example, and to the co-development process in general were identified. We further conclude that review of the final version of the FDA guidance document on in vitro companion diagnostics as well as additional specific examples will be important to determine whether the co-development process will be sufficient to ensure adequate evidence of clinical utility for all companion diagnostics approved via this regulatory review pathway.
BRAF Gene Mutation Testing to Select Patients with Melanoma for Treatment with BRAF Kinase Inhibitors

Objective

Drug discovery and development generally follows one of two different paradigms: physiologic-based discovery or target based-discovery. Physiologic-based drug discovery screens a large number of compounds using a physiologic readout such as an animal model or a cellular assay. Target-based drug discovery begins by identifying the function of a possible therapeutic target, such as an enzyme or a cell surface receptor, and its role in disease. Particularly in the latter case, patients often must be selected for treatment using a laboratory test (“companion diagnostic”) that identifies which patients have the target.

Examples of target-based discovery include imatinib, originally designed to inhibit the constitutively activated kinase product of the abnormal fusion gene BCR-ABL found in chronic myelogenous leukemia (CML); and trastuzumab, an antibody designed to bind to HER2 receptors overexpressed on the cell surface of some types of breast cancer. In the case of imatinib, however, as nearly all patients with CML harbor the BCR-ABL gene, a companion test to initially select patients for treatment is largely unnecessary. The HER2 companion test initially commercialized was not used in the clinical trials of trastuzumab, and the subjectivity component of test readout was not initially appreciated, resulting in the design of improved HER2 tests and extended analyses of the quality and utility of HER2 companion testing. In some cases, companion test development and evaluation is retrospective, as in the case of KRAS mutation testing to select colorectal cancer patients likely to respond to cetuximab or panitumumab treatment. Thus, these are not the best examples of treatment-test co-development.

Recent examples of targeted drug design and companion test co-development include crizotinib (Pfizer, Inc.), which targets an abnormal fusion gene product in some patients with non-small cell lung cancer, and vemurafenib (Hoffmann-La Roche Ltd.) and dabrafenib (GlaxoSmithKline plc), both BRAF inhibitors that target a mutated form of the BRAF kinase, derived from the mutated BRAF gene (BRAF(V600E)) occurring in 40–60% of melanoma tumors. This mutation results in a change from valine to glutamic acid at amino acid position 600 in the BRAF protein. As these products were in development, the U.S. Food and Drug Administration (FDA) was developing a draft guidance for in vitro companion diagnostics, the major principles of which were likely applied to products nearing FDA submission, including crizotinib and vemurafenib. This guidance is important not only for its applicability to targeted drug development, but also for its impact on a broader category of “personalized” treatments that use a companion test to select patients most likely to respond, or to avoid treating patients likely to have serious adverse reactions.

An important question is whether the co-development process ensures sufficient validation of the companion diagnostic test, such that an independent evaluation becomes unnecessary. This Report will address that question using a targeted drug discovery example: the recently approved vemurafenib for certain patients with melanoma and its co-approved companion diagnostic test.

Background

FDA Guidance on Companion Diagnostics

The FDA Centers for Devices and Radiological Health (CDRH), for Biologics Evaluation and Research (CBER), and for Drug Evaluation and Research (CDER) developed a draft guidance on in vitro companion diagnostic devices, released on July 14, 2011 (FDA 2011). The FDA has extended to October 12, 2011, the comment period for the draft guidance document entitled “In Vitro Companion Diagnostic Devices.” In the announcement in the Federal Register, the Agency noted it is taking this action in response to requests for an extension to allow interested persons additional time to submit comments.
While the guidance is not yet finalized, it represents the FDA’s current thinking on the topic and likely the direction given to sponsors of applicable treatments and companion diagnostics in development at the same time this guidance was being prepared. The main points for new companion diagnostic tests and new therapeutic products are summarized from the guidance as follows:

- Because results from the companion diagnostic test are a determining factor in patient treatment, the FDA will assess the safety and effectiveness of the test. At the time of this writing, the FDA does not exercise regulatory oversight with regard to laboratory-developed tests (LDT), and guidance on such regulation is not planned until 2012 at the earliest.4 Thus, one interpretation is that LDTs are not acceptable companion diagnostic tests if submitted with the therapeutic product for approval.
- FDA intends to review each companion diagnostic in conjunction with its corresponding therapeutic product, and review of each product pair will be conducted collaboratively among relevant offices.
- A new therapeutic product and its corresponding companion diagnostic test should be developed together, with the clinical performance and significance of the companion diagnostic test established using data from the clinical development program of the corresponding therapeutic product.
- FDA will apply a risk-based approach early in discussion to determine whether a companion diagnostic test requires premarket approval (PMA) or 510(k) clearance.
- If use of a companion diagnostic test is essential for the safe and effective use of a new therapeutic product, both diagnostic test and therapeutic product should be approved or cleared at the same time by the FDA (rare exceptions possible).
- If the companion diagnostic is not approved or cleared for the indication for which the new therapeutic product is submitted, the therapeutic product will not be approved (rare exceptions possible).
- The use of a cleared or approved companion diagnostic test with a particular approved therapeutic product must be stated in the labeling of both diagnostic test and corresponding therapeutic product. Only the type of companion diagnostic is described, not a particular manufacturer’s test.
- Marketing a cleared or approved companion diagnostic and its corresponding therapeutic product for a new use represents a major change in intended use and requires a new PMA or 510(k) submission.
- New versions of a companion diagnostic test (e.g., developed by a different manufacturer) intended to perform the same function as an existing approved or cleared companion diagnostic will be reviewed under a PMA or 510(k), as appropriate. Thus, competitor companion diagnostic tests are provided an avenue for FDA clearance or approval. No mention is made of laboratory-developed tests.

Developing Targeted Treatment: BRAF Inhibitors for Melanoma

Advanced/Metastatic Melanoma. Overall incidence rates for melanoma have been increasing for at least 30 years; in 2011, more than 70,000 new cases will be diagnosed (ACS 2011). In advanced (Stage IV) melanoma, the disease has spread beyond the original area of skin and nearby lymph nodes. For the small proportion of cases that are Stage IV at diagnosis, prognosis is extremely poor; 5-year survival is about 15–20%. Prognosis is somewhat variable depending on whether disease has spread to other organs and whether blood lactate dehydrogenase remains normal.

Fully disseminated Stage IV disease that has also spread to the brain may be treated with systemic therapy, resection and/or radiation, and/or best supportive care. Stage IV disease that is limited and resectable may be treated with systemic therapy after surgery, choosing from the same treatment options recommended for nonresectable disease, as follows (NCCN 2011):

- Clinical trial (preferred)
- Ipilimumab (potential for significant immune-mediated complications)
- Dacarbazine
- Temozolomide
- High-dose interleukin-2 (potential for significant toxicity)
- Dacarbazine- or temozolomide-based combination therapy
- Paclitaxel single-agent or combination therapy

---

4 See Documents the Center for Devices and Radiological Health is Considering for Development (FY12) at http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/Overview/MedicalDeviceUserFeeandModernizationActMDUFMA/ucm109196.htm
Dacarbazine has long been considered the treatment standard for systemic therapy, but has disappointingly low response rates of only 15 to 25% and median response durations of 5 to 6 months; less than 5% of responses are complete (Gogas et al. 2007). Temozolomide has similar efficacy with the exception of a much greater ability to penetrate the central nervous system. Combination regimens increase response rates, but not overall survival.

Treatment recommendations for patients with advanced melanoma changed recently with the approval of ipilimumab by the U.S. Food and Drug Administration (FDA) in March 2011 for the treatment of patients (previously treated or not) with unresectable or metastatic melanoma. For the first time, a survival advantage was demonstrated in previously treated patients: median survival on ipilimumab of 10 months versus 6.4 months on control medication (Table 1). As noted earlier, ipilimumab is the first recommended treatment choice if the patient is not enrolled in a clinical trial. New treatments, including those in development while ipilimumab was being reviewed and approved (e.g., vemurafenib), must be considered in relation to ipilimumab. A disadvantage of ipilimumab, which augments T-cell activation and proliferation, presumably increasing anti-tumor immune responses, is the possibility of severe and fatal immune-mediated adverse reactions, especially in patients who are already immunocompromised.

Table 1. Ipilimumab Pivotal Trial Results (Hodi et al. 2010)

<table>
<thead>
<tr>
<th></th>
<th>Ipilimumab + gp100 Vaccine (n=403)</th>
<th>Ipilimumab-only (n=137)</th>
<th>gp100 Vaccine-only (n=136)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Deaths</td>
<td>306</td>
<td>100</td>
<td>119</td>
</tr>
<tr>
<td>Overall Survival</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hazard ratio (95% CI) vs. gp100 only</td>
<td>0.68 (0.55, 0.85)</td>
<td>0.66 (0.51, 0.87)</td>
<td>—</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>—</td>
</tr>
<tr>
<td>Progression-Free Survival, Median, months (95% CI)</td>
<td>2.76 (2.73, 2.79)</td>
<td>2.86 (2.76, 3.02)</td>
<td>2.76 (2.73, 2.83)</td>
</tr>
<tr>
<td>Best Overall Response Rate, % (95% CI)</td>
<td>5.7 (3.7, 8.4)</td>
<td>10.9 (6.3, 17.4)</td>
<td>1.5 (0.2, 5.2)</td>
</tr>
</tbody>
</table>

5 Conventionally, gene symbols are italicized, with all letters in uppercase. Protein designations are the same as the gene symbol, but are not italicized. See http://www.genenames.org/guidelines.html.

6 Kinases are enzymes that transfer phosphate groups from high-energy donor molecules, such as adenosine triphosphate (ATP) to specific substrates in a process called phosphorylation. Kinases are used extensively to transmit signals and control complex processes in cells. Over 500 different kinases have been identified in humans (Manning et al. 2002).

BRAF Mutations. In 2002, Davies et al. reported that 66% of cutaneous malignant melanomas had somatic mutations in the BRAF5 gene at the V600E locus (a change from valine to glutamic acid at amino acid position 600 in the BRAF protein; gene mutation designated BRAF V600E in this report, wild-type or normal gene designated BRAF WT) (Davies et al. 2002). BRAF codes for a kinase5 component in the RAF-MEK-ERK signal transduction phosphorylation cascade within the cellular mitogen-activated protein kinase (MAPK) pathway. It was already known that the MAPK signaling pathway becomes constitutively activated in the majority of melanomas, promoting tumor proliferation, invasion, and angiogenesis (Vultur et al. 2011).

BRAF V600E mutations are the most common in this gene, although other substitutions besides glutamic acid have been identified at this locus, and mutations at other loci in the kinase domain have also been described. Most BRAF kinase domain mutations increase the kinase activity (Ribas and Flaherty 2011); high-activity mutations such as V600E increase the kinase activity 500-fold compared to wild type and provide one explanation for constitutive MAPK pathway activity (Solit et al. 2006). V600 mutations also appear to be mutually exclusive with other mutations in the MAPK pathway such as activating mutations in KIT and NRAS; on the basis of this and other data, melanoma tumors with V600 mutations are believed to be dependent on RAF-MEK-ERK signaling for oncogenic proliferation (Ribas and Flaherty 2011).
In general, 50-70% of melanoma tumors harbor a BRAF mutation and of these, 80% are positive for BRAF^{V600E} (Vultur et al. 2011), suggesting a natural target for cancer inhibition. Early studies of advanced melanoma treatment with the nonselective kinase inhibitor sorafenib were not successful (Eisen et al. 2006; Hauschild et al. 2009). Similarly, inhibitors of MEK, another possible target in the same signaling pathway, were only minimally successful in clinical trials (Dummer et al. 2008). However, studies of short interfering RNA that prevented mutated BRAF expression in xenograft murine models resulted in tumor regression (Hoeﬁlch et al. 2006), leading to the design of highly selective inhibitors of mutated BRAF kinase.

**Designing a Targeted BRAF^{V600E} Inhibitor.**

Two companies developed targeted BRAF inhibitors that have proceeded to Phase III clinical trials in melanoma patients. Vemurafenib (trade name Zelboraf®, also known as PLX4052 and RO5185426) was co-developed under an agreement between Roche (Genentech) and Plexxikon. Vemurafenib and a companion diagnostic test developed by Roche Molecular Systems have been approved by the FDA. Dabrafenib (also known as GSK2118436 or SB-590885) is a BRAF inhibitor developed by GlaxoSmithKline (King et al. 2006; Takle et al. 2006) and is currently in clinical trials. However, as few publications detailing preclinical or clinical studies for dabrafenib are available, and neither drug nor companion test (developed by bioMérieux) have as yet been submitted to the FDA, the rest of this Report will focus on vemurafenib.

**Summary.** Vemurafenib was developed using a fragment-based, structure-guided approach that allowed the synthesis of a compound with high potency to inhibit the BRAF V600E mutated kinase, and signiﬁcantly lower potency to inhibit most of many other kinases tested. Preclinical studies demonstrated that vemurafenib selectively blocked the RAF/MEK/ERK pathway in BRAF mutant cells and caused regression of BRAF mutant human melanoma xenografts in murine models. Paradoxically, preclinical studies also showed that BRAF<sup>WT</sup> melanoma tumors could respond to mutant BRAF-speciﬁc inhibitors with accelerated growth, suggesting that it might be harmful to administer BRAF inhibitors to patients with BRAF<sup>WT</sup> melanoma tumors.

For the interested reader, additional detail on the design and preclinical testing of vemurafenib is provided in this and the next section, including the molecular and preclinical data supporting the selectivity of vemurafenib and a discussion of the enrichment design used in the vemurafenib clinical trials. Readers not desiring this detail should continue to the section entitled, “Regulatory Pathway.”

**Drug Design.** Vemurafenib was originally designated PLX4052 in the published literature and is a member of a family of kinase inhibitors designated for that purpose. Vemurafenib is an analog of PLX4720; the design process for PLX4720 has been published in detail (Tsai et al. 2008). Briefly, a fragment-based discovery approach (Jhoti 2005) was used in which much lower molecular weight compounds than those typically found in high-throughput compound collections were used for screening. Initially, 258 of 20,000 low-molecular-weight fragments (also called scaffolds) were found to inhibit 5 test kinases. These fragments were crystallized bound to at least one kinase and more than 100 molecular structures were solved by crystallographic analysis. This allowed selection of one fragment with the most appropriate binding and affinity for the ATP binding site of the kinase.

Based on this fragment, a group of related compounds with increased kinase afﬁnity were synthesized for optimization by structure-guided synthesis and characterization of analogs. This process allowed for a rapid deﬁnition of the key molecular interactions necessary for binding to the ATP site of the kinase, and synthesis of analogs with increased potency and selectivity, including PLX4720 and PLX4052. The crystal structure of the BRAF kinase/PLX4720 complex was solved and PLX4720 was found to preferentially bind to one of two conformations of the wild-type BRAF kinase and to the sole and similar conformation of the BRAF<sup>V600E</sup> mutant kinase. These and other results supported the conclusion that PLX4720 preferentially binds the active form of the BRAF kinase (and that the V600E mutation “locks” the BRAF kinase into the active conformation, thus explaining constitutive activation). The active conformation of the BRAF kinase contains a “Raf-selective pocket” that binds a propyl group on PLX4720. Comparison with many other kinase structures showed that few kinases contained this pocket at the same conformational location; thus,
PLX4720 access to this conformational pocket plays a role in kinase selectivity.

Tsai et al. (Tsai et al. 2008) document PLX4720 selectivity for the \(BRAF^{V600E}\) mutant kinase in melanoma cell lines and in direct examination of the Raf-MEK-ERK signal transduction cascade output (phosphorylation) in cells bearing or not bearing the \(BRAF^{V600E}\) mutation, concluding that PLX4720 has anti-melanoma activity only against cells that harbor the V600E mutation. Although PLX4720 was described in detail early in development, it was a first-generation inhibitor from which the analog PLX4052 was subsequently derived (Lee et al. 2010). The inhibitory properties of PLX4052 were also characterized in detail and PLX4052 was eventually chosen over PLX4720 “because its pharmacokinetic properties scaled appropriately in beagle dogs and cynomolgus monkeys” (Bollag et al. 2010).

**Preclinical Development.** PLX4052 (renamed RG7204, then later generically named vemurafenib; for the rest of this section, referred to as PLX4052) was also co-crystallized with the kinase domain of \(BRAF^{V600E}\) and was found to bind in the same way as PLX4720. PLX4052 was tested for its ability to inhibit the activity of over 200 kinases; it has similar potency for kinases coded by the genes \(BRAF^{V600E}, RAFL, SRMS, TNK2, MAP4K5,\) and \(FGR.\) Other kinases tested required significantly greater concentrations of PLX4052 to inhibit 50% of the kinase activity in vitro (IC\(_{50}\)), indicating much lower potency. Appendix Table A lists the kinases for which PLX4052 had the greatest inhibitory activity and their general functions, if known; there was minimal activity against all others tested (Bollag et al. 2010). Although of similar potency to the \(BRAF^{V600E}\)-coded kinase in in vitro assays, PLX4052 inhibition of kinases coded by \(RAFL, SRMS, TNK2, MAP4K5,\) and \(FGR\) has not been tested in cellular assays (Bollag et al. 2010). Whether other cofactors that typically interact with kinases may interfere with the non-\(BRAF^{V600E}\) kinase inhibitory reactions is unknown.

When tested in vitro against many cancer cell lines by different groups of researchers, PLX4052 was a potent inhibitor of proliferation in cell lines expressing \(BRAF^{V600E}\) as well as in cell lines expressing other \(BRAF\) V600 mutant kinases (Joseph et al. 2010; Sondergaard et al. 2010; Yang et al. 2010). However, the inhibitory response is not quantitatively uniform; Lee et al. report that the IC\(_{50}\) ranges from low nanomolar to nearly 10 micromolar across different cell lines, suggesting that mutant BRAF inhibition is not the sole reaction affecting proliferation (Lee et al. 2010). In cell lines from other tumor types, PLX4052 did not inhibit proliferation except for a breast cancer cell line that expresses \(BRAF^{V600E}\). PLX4052 effected cell cycle arrest in cell lines expressing \(BRAF^{V600E}\) but not in \(BRAF^{WT}\) cells; nor was growth in \(BRAF^{WT}\) cells promoted (Joseph et al. 2010; Yang et al. 2010).

PLX4052 suppressed Raf-MEK-ERK signaling in all \(BRAF^{V600E}\)-expressing cell lines and in cell lines expressing the \(BRAF^{V600D}\) and \(BRAF^{V600R}\) mutations (Joseph et al. 2010; Sondergaard et al. 2010; Yang et al. 2010); the effect on \(BRAF^{WT}\) cell lines, however, was more variable. In some \(BRAF^{WT}\) cell lines, there was no PLX4052 inhibition as expected, but in others as well as in normal keratinocyte cultures there was evidence of rapid and markedly increased MAPK pathway signaling over a range of PLX4052 concentrations (Joseph et al. 2010; Sondergaard et al. 2010; Yang et al. 2010), which is believed to be due to a loss of negative regulatory feedback. Joseph et al. found this to be a common property of several ATP-competitive inhibitors of RAf-kinases (Joseph et al. 2010). This has raised concern that PLX4052 treatment in patients with \(BRAF^{WT}\) tumors could potentiate tumor growth. As already noted, assays of \(BRAF^{WT}\) cell lines did not show cell cycle G1 progression or proliferation in response to treatment with PLX4052. However, additional work has further clarified the molecular mechanism of this paradoxical Raf-MEK-ERK activation (Hatzivassiliou et al. 2010; Heidorn et al. 2010; Poulikakos et al. 2010). The results suggest not only that \(BRAF^{WT}\) melanoma tumors could respond to mutant BRAF-specific inhibitors with accelerated growth; but also that relatively rare melanoma tumors that are positive for \(BRAF^{V600E}\) and that co-express mutant RAS may be resistant to mutant BRAF-specific inhibitors.

In vitro models attempted to model tissue architecture, first by embedding melanoma spheroids prepared from cell lines and from freshly harvested human melanoma tumor specimens into a collagen matrix. Matrices were overlayed with increasing concentrations of PLX4052; only \(BRAF^{V600E}\)-positive
tumor growth was inhibited (Lee et al. 2010). Similarly-treated melanoma skin reconstructs also resulted in $BRAF^{V600E}$-specific growth inhibition (Lee et al. 2010).

PLX4052 inhibited the growth of $BRAF^{V600E}$-mutant xenograft tumors in mice in a dose-dependent fashion, with higher exposures resulting in complete tumor regression. Efficacy was demonstrated whether $BRAF$ mutations were heterozygous or homozygous (Bollag et al. 2010). PLX4052 had greatly improved antitumor activity compared to the maximum tolerable dose of temzolomide against mutant xenograft tumors; PLX4052-treated mice had a $61\%$ increased life span. In contrast, when both $BRAF$ alleles were wild-type, PLX4032 had no inhibitory effect on xenografts. Additional studies in mice, rats, dogs, and monkeys provided benchmarks of exposure and toxicity in preparation for human clinical studies.

Regulatory Pathway. The developmental and preclinical work on PLX4720 and PLX4052/vemurafenib demonstrated high selectivity of the inhibitors for the active and mutated form of the $BRAF$ kinase, strong preferential binding versus the wild-type form of $BRAF$, and low potency for most of many other kinases tested. Treatment of melanoma xenografts in murine models with vemurafenib suggested strong $BRAF^{V600E}$-specific responses with a significant improvement in survival. In addition, the variable response of $BRAF^{WT}$ cell lines to the analogs suggested that treatment of $BRAF^{WT}$ tumors might have adverse effects, possibly including potentiation of tumor growth. These were likely important considerations for the choice of a drug-diagnostic development regulatory pathway in which a diagnostic test would be developed and used to select patients with $BRAF^{V600E}$-positive melanoma tumors for the pivotal clinical studies. This would mean that approximately $40-60\%$ of melanoma patients would be eligible for the drug, if approved.

Thus, all or most clinical studies of vemurafenib were “enrichment studies” in which only patients with the drug target, and therefore likely to respond, were treated. Patients without the drug target, unlikely to respond and in this case with hypothesized potential for incurring harm, were not enrolled. The major advantages of such trials are smaller size, faster time to completion, and overall greater efficiency; in the FDA’s Critical Path white paper of 2004, enrichment designs were identified as having the potential to provide much earlier assurance of drug activity (FDA 2004). The enrichment design is most appropriate for situations in which there is a strong biological basis supporting the nonresponse of patients without the drug target, and thus including them might be unethical, or evidence suggesting the potential for harm (Simon 2008). The design and preclinical data for vemurafenib appear to meet these criteria.

The advantages of the enrichment design are primarily practical rather than scientific. A disadvantage is the loss of information regarding the treatment effect on patients with $BRAF^{WT}$ melanoma tumors. Despite the signal of potential harm from preclinical studies, carefully studying the actual effect of vemurafenib might be valuable to the significant proportion of $BRAF^{WT}$ advanced melanoma patients who may have few other treatment choices. Thus, the result of the co-development process is necessarily a narrow treatment indication.

Critical to the design of the clinical trials to determine dose, safety, and ultimately the effectiveness of vemurafenib was the early development of a diagnostic test to reliably select patients with melanoma tumors positive for $BRAF^{V600E}$. Whether the co-development process ensures sufficient analytic and clinical validation of the companion diagnostic, using the test for $BRAF^{V600E}$ as an example, is the topic of this Report.

Methods

Search Methods
The MEDLINE® database was searched (via PubMed) for articles using the terms “PLX4052,” “vemurafenib,” “V600E,” and “BRAF inhibitor,” all coupled with the term “melanoma.” The reference lists of relevant study publications and review articles were also examined. The meeting abstracts for the 2011 annual meeting of the American Society of Clinical Oncology were searched using the MEDLINE® search terms. If available, virtual presentations and slides were reviewed for key abstracts. The “grey literature” was consulted in the form of drug and laboratory test approval information released by the FDA, ongoing clinical trials from www.clinicaltrials.gov, and online searches for status and ancillary information.
**Study Selection**

For primary clinical data, we looked for trials of vemurafenib that used the final version of the companion test to select patients. Trials that used a prototype assay were also considered.

**Medical Advisory Panel Review**

This Special Report was reviewed by the Blue Cross and Blue Shield Association Medical Advisory Panel (MAP) on September 8, 2011. To maintain the timeliness of the scientific information in this Special Report, literature search updates were performed subsequent to the Panel’s review (see “Search Methods”). If the search updates identified any additional studies that met the criteria for detailed review, the results of these studies were included in the text where appropriate. There were no studies that would change the conclusions of this Report.

**Review**

In May 2011, Roche Molecular Systems filed a premarketing application (PMA) with the Office of In Vitro Diagnostics (OIVD), Center for Devices and Radiological Health, at the FDA for approval of the cobas® 4800 BRAF V600 Mutation Test as a companion diagnostic test with vemurafenib. PMAs are required for Class III devices and require a high level of support data. The test was co-filed with vemurafenib, which was submitted for approval to the FDA Center for Drug Evaluation and Research. Both test and drug were co-approved, each by the appropriate FDA Center, on August 17, 2011.

The full prescribing information for vemurafenib (Zelboraf®) states that “BRAF V600E testing – confirmation of BRAF V600E mutation using an FDA-approved test is required for selection of patients appropriate for ZELBORAF therapy” and notes that the efficacy and safety of vemurafenib have not been studied in patients with melanoma tumors that have BRAF WT. The kit insert for the Roche BRAF V600 Mutation Test notes that the assay is designed to detect the V600E mutation and that the test is intended to be used as a companion diagnostic for vemurafenib.

This review will examine the evidence generated for the companion diagnostic and drug submission and approval process, using the cobas® 4800 BRAF V600 Mutation Test and vemurafenib as an example, to ask whether the process is sufficient for validation of companion diagnostic tests in general. The components of the evidence evaluation are analytic validity, clinical validity, and clinical utility, as defined in the methods of the Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group (Teutsch et al. 2009).

1. What is the evidence supporting the analytic validity of the cobas® 4800 BRAF V600 Mutation Test?

“The analytic validity of a genetic test [is] its ability to accurately and reliably measure the genotype (or analyte) of interest in the clinical laboratory, and in specimens representative of the population of interest. Analytic validity includes analytic sensitivity (detection rate), analytic specificity (1–false positive rate), reliability (e.g., repeatability of test results), and assay robustness (e.g., resistance to small changes in preanalytic or analytic variables)” (Teutsch et al. 2009).

Submission to the Office of In Vitro Diagnostics of the FDA for marketing clearance or approval of a diagnostic test requires an extensive demonstration of the analytic validity of the test. Data for cleared or approved tests are summarized in the kit insert (prepared by the manufacturer) and in the Summary of Safety and Effectiveness of the test (prepared by the FDA and publicly available).

The cobas® 4800 BRAF V600 Mutation Test is a real-time polymerase chain reaction (PCR) test intended for the qualitative detection of the BRAF V600E mutation specifically in DNA that has been extracted from formalin-fixed, paraffin-embedded (FFPE) human melanoma tissue. Data correlating the results of the test to the reference standard of Sanger bidirectional sequencing are summarized below; additional details regarding the test and an abbreviated summary of the other major components of the analytic validation are provided in the Appendix (Validation of the cobas® 4800 BRAF V600 Mutation Test).

One limitation was lack of complete data for calculating sensitivity and specificity. Sanger sequencing was used as a gold standard for FDA submission studies, but due to insensitivity at lower percentages of mutated alleles (<20%) is an inappropriate standard. For the Phase III trial, samples from patients screened for the trial were sequenced, including those
which were \( \text{BRAF}^{\text{WT}} \); for the Phase II trial only enrolled patients were sequenced, thus only percent agreement between the two methods could be calculated. Subsequent studies indicated a high percent agreement between the cobas 4800 BRAF V600 Mutation Test and more sensitive sequencing methods but insufficient information was available to allow recalculation of the Phase III sample data. Comparison with sequencing showed that in addition to very high percent agreement for the V600E mutation, the companion test also identified some, but not all, V600 variant mutations (e.g., V600K) as positive. Tumors with V600 variant mutations have a different amino acid substitution in the BRAF kinase. Performance characteristics of the companion test varied if the definition of a positive result was changed (Table 2).

Summary. Correlation with sequencing and extensive analytic validation data support a sensitive and robust assay for the detection of the V600E mutation in FFPE melanoma specimens. Additionally, some, but not all patients with V600 mutations other than V600E will also be identified as positive. The test is available as a kit and is partially automated, which should result in wide access and rapid turnaround time relative to the reference standard of sequencing.

2. What is the evidence supporting the clinical validity of the cobas® 4800 BRAF V600 Mutation Test?

5. What is the evidence supporting the clinical utility of the cobas® 4800 BRAF V600 Mutation Test?

Table 2. Correlation of Vemurafenib Trial Companion Test Results with Sanger Sequencing

<table>
<thead>
<tr>
<th>Definition of Positive</th>
<th>Positive % Agreement</th>
<th>Negative % Agreement</th>
<th>Overall % Agreement</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phase III trial (Chapman et al. 2011)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Only V600E</td>
<td>97.3</td>
<td>84.6**</td>
<td>90.9</td>
<td>97</td>
<td>85</td>
</tr>
<tr>
<td>All V600</td>
<td>87.7</td>
<td>95.4**</td>
<td>90.6</td>
<td>88</td>
<td>95</td>
</tr>
<tr>
<td>V600E + V600K</td>
<td>92.7</td>
<td>95.2**</td>
<td>91.1</td>
<td>93</td>
<td>95</td>
</tr>
<tr>
<td><strong>Phase II trial (Bloom et al. 2011)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Only V600E</td>
<td>92.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V600E + V600K</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


**Sanger sequencing identified

“The clinical validity of a genetic test [is] its ability to accurately and reliably predict the clinically defined disorder or phenotype of interest. Clinical validity encompasses clinical sensitivity and specificity (integrating analytic validity), and predictive values of positive and negative tests that take into account the disorder prevalence (the proportion of individuals in the selected setting who have, or will develop, the phenotype/clinical disorder of interest)” (Teutsch et al. 2009).

“The clinical utility of a genetic test [is] the evidence of improved measurable clinical outcomes, and its usefulness and added value to patient management decision-making compared with current management without genetic testing” (Teutsch et al. 2009).
When a treatment is developed for a specific biological target that characterizes only some patients with a particular disease, and a test is co-developed to identify diseased patients with that target, clinical validity and clinical utility studies are no longer separate and sequential. Rather, the clinical studies of treatment benefit, which use the test to select patients, provide evidence of both clinical validity and clinical utility. The primary evidence of clinical validity and utility for the cobas® 4800 BRAF V600 Mutation Test is provided by the Phase III clinical trial of vemurafenib. In addition, evidence from Phase I and Phase II trials will also be reviewed. As previously noted, all trials were enrichment trial designs, in which all patients were positive for a V600 mutation (with a few exceptions in the Phase I trial).

**Phase I Clinical Trial.** The major goals of this trial were to first determine the maximum dose in a dose-escalation phase, then determine the objective response rate and monitor toxicity (Flaherty et al. 2010). This trial used a PCR assay that was likely a prototype of the final test; only a brief description of the assay was provided in the publication. To determine response, computed tomography (CT) studies were performed at 8-week intervals in all patients, and after 4 weeks of therapy in some. CT results were judged according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.0.7

**Dose-escalation Phase.** Although patients were not required to have BRAFV600E-positive melanoma for this part of the trial, selection was weighted in favor of the mutation. Sixteen patients with V600E mutation-positive metastatic melanoma were dosed at 240 to 1,120 mg vemurafenib twice daily. Main results were as follows:

- Eleven of 16 patients responded (69%; 10 partial, 1 complete response).
- The duration of response was 2 to 18 months or more.
- Of 5 patients with metastatic melanoma tumors who did not have the V600E mutation and who received 240 mg or more PLX4052 twice daily, none responded.

- A dose of 960 mg twice daily was tolerated well and was the recommended dose for the extension phase of the trial.

**Extension Phase.** Patients in this phase of the trial were required to have BRAFV600E-positive melanoma according to the test used. Thirty-two patients with BRAFV600E-positive metastatic melanoma tumors were dosed at the recommended 960 mg vemurafenib twice daily (or less if toxicity). Main results were as follows:

- Twenty-six of 52 patients responded (81%; 24 partial, 2 complete responses).
- Symptom improvement was reported within 1–2 weeks.
- Responses were seen in visceral organs and bone, as well as in typical sites of improvement such as lungs and lymph nodes.
- Estimated median progression-free survival was greater than 7 months by Kaplan-Meier analysis; estimated overall survival was not reached at time of publication.

**Phase II Clinical Trial (ongoing).** The goal of this Phase II trial, also known as BRIM-2, was to confirm the response rate and anti-tumor activity of vemurafenib in patients with histologic Stage IV melanoma but no active metastases to the central nervous system (CNS), who had been previously treated. The study was conducted at 15 centers, 10 in the U.S. and 5 in Australia. All patients were selected with the cobas 4800 BRAF V600 Mutation Test; 122 cases had BRAFV600E-positive melanoma, and 10 cases were positive for BRAFV600K. The early results of this trial are available only as a meeting abstract and a meeting slide presentation (Ribas et al. 2011).

All 132 enrolled patients received 960 mg vemurafenib orally twice daily until progressive disease, unacceptable toxicity, or death. The primary outcome of the trial was best overall response rate, as evaluated by an independent review committee. The target overall response rate was 50%, with a lower boundary of the 95% confidence interval (CI) of at least 20%. At a median follow-up of 10 months, this target was met with an overall response rate of 55% (95% CI: 44–62%; see Table 5). At 10 months,

---

7 A complete response is defined as the disappearance of all target lesions. A partial response is defined as a decrease by at least 50% in the sum of the largest diameter of each target lesion, relative to the corresponding sum at baseline. Progressive disease is defined as an increase by at least 20% in the sum of the largest diameter of each target lesion, relative to the smallest corresponding diameter recorded since the start of treatment, or the appearance of one or more new lesions. Stable disease is defined as an absence of shrinkage sufficient for a partial response and the absence of enlargement sufficient for progressive disease, relative to the corresponding sum at baseline.
27% of patients were still on treatment; the majority of discontinuations were due to disease progression. The most common adverse events of any grade were arthralgias (58%), skin rash (52%), and photosensitivity (52%). The most common grade 5 adverse event was squamous cell carcinomas, which were seen in about 25% of patients, tended to occur in the first 2 months of treatment, and were managed with local excision. There were very few grade 4 adverse events.

**Phase III Clinical Trial.** This comparative trial, also known as BRIM-5, randomly assigned 675 patients to either vemurafenib (960 mg twice daily orally) or dacarbazine (1,000 mg/m² body surface area by IV infusion every 3 weeks) to determine whether vemurafenib would prolong the rate of overall or progression-free survival, compared to dacarbazine (Chapman et al. 2011). All enrolled patients had unresectable, previously untreated Stage IIIIC or IV melanoma with no active CNS metastases. Melanoma specimens from all patients tested positive for the BRAF<sup>V600E</sup> mutation on the cobas 4800 BRAF V600 Mutation Test. Included were 19 patients with BRAF<sup>V600K</sup> mutations and one with a BRAF<sup>V600D</sup> mutation.

Tumor assessments including CT were performed at baseline, at weeks 6 and 12, and every 9 weeks thereafter. Tumor responses were determined by the investigators according to the RECIST, version 1.1. The original primary endpoint was the rate of overall survival; after Phase I and II trial results were known, progression-free survival was added as a co-primary endpoint. An interim analysis was planned at 98 deaths and a final analysis at 196 deaths; the published report is the interim analysis, reporting 118 deaths (Table 4).

The median survival had not been reached; the hazard ratio for death was 0.37 (95% CI: 0.26–0.55). At 6 months, overall survival was 84% (95% CI: 78 to 89) for vemurafenib-treated patients and 64% (95% CI: 56 to 75) for dacarbazine-treated patients. Progression-free

---

**Table 3. Phase II Vemurafenib Single-arm Study (BRIM-2)**

<table>
<thead>
<tr>
<th></th>
<th>Vemurafenib</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=132)</td>
<td></td>
</tr>
<tr>
<td>Overall Survival</td>
<td></td>
</tr>
<tr>
<td>Median, months (95% CI)</td>
<td>Not Reached</td>
</tr>
<tr>
<td>6 months</td>
<td>77% (95% CI: 70–85%)</td>
</tr>
<tr>
<td>12 months</td>
<td>58% (95% CI: 49–67%)</td>
</tr>
<tr>
<td>Progression-Free Survival</td>
<td></td>
</tr>
<tr>
<td>Median, months</td>
<td>6.7 (95% CI: 5.5–7.8)</td>
</tr>
<tr>
<td>6 months</td>
<td>54% (95% CI: 45–63%)</td>
</tr>
<tr>
<td>Response</td>
<td></td>
</tr>
<tr>
<td>Objective Response Rate</td>
<td>53% (95% CI: 44–62%)</td>
</tr>
</tbody>
</table>

**Table 4. Phase III Trial Comparing Vemurafenib to Dacarbazine (BRIM-3)**

<table>
<thead>
<tr>
<th></th>
<th>Vemurafenib</th>
<th>Dacarbazine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Survival, n evaluable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median, months (95% CI)</td>
<td>336</td>
<td>336</td>
</tr>
<tr>
<td>6 months</td>
<td>Not Reached</td>
<td>Not Reached</td>
</tr>
<tr>
<td>Hazard ratio (95% CI)</td>
<td>84% (95% CI: 78–89%)</td>
<td>65% (95% CI: 56–73%)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.37 (95% CI: 0.26–0.55)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Progression-Free Survival, n evaluable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median, months</td>
<td>275</td>
<td>274</td>
</tr>
<tr>
<td>Hazard ratio (95% CI)</td>
<td>5.3</td>
<td>1.6</td>
</tr>
<tr>
<td>p-value</td>
<td>0.26 (95% CI: 0.20–0.33)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Response, n evaluable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Objective Response Rate, % (95% CI)</td>
<td>219</td>
<td>220</td>
</tr>
<tr>
<td>48% (95% CI: 42 to 55%)</td>
<td>5% (95% CI: 3 to 9%)</td>
<td></td>
</tr>
</tbody>
</table>
survival was evaluable in 549 patients; the hazard ratio for tumor progression was 0.26 (95% CI: 0.20–0.33). The median progression-free survival was estimated to be 5.3 months for patients treated with vemurafenib and 1.6 months for patients treated with dacarbazine. Tumor response was evaluable in 439 patients; the objective response rate was 48% in patients treated with vemurafenib versus 5% in those treated with dacarbazine. Only 2 patients treated with vemurafenib had a complete response. Of the 10 patients with V600K-positive tumors randomized to vemurafenib treatment, 4 had partial responses. Adverse events in the vemurafenib group included grade 2 or 3 photosensitivity skin reactions in 12% of patients, and cutaneous squamous cell carcinoma in 18% of patients. The data and safety monitoring board determined that both co-primary endpoints had met prespecified criteria for statistical significance and recommended that patients in the dacarbazine group be allowed to cross over and receive vemurafenib.

The results of this trial comprised the data supporting the efficacy and safety of vemurafenib for submission to the FDA, and established the safety and effectiveness of the cobas 4800 BRAF V600 Mutation Test (see Appendix for links to relevant documents), resulting in co-approval of drug and companion test.

During the course of the Phase III trial, ipilimumab was approved for the treatment of unresectable or metastatic melanoma and became a favorable treatment choice. Because the median survival was not reached in the vemurafenib trial, no comparison can be made on this outcome. However, objective response rate was 48% for vemurafenib and 11% for ipilimumab in separate trials; median progression-free survival was 5.3 months for vemurafenib and 2.9 months for ipilimumab.

**Summary.** The results of the Phase III trial, supported by the results of the earlier trials, support the clinical validity and clinical utility of the cobas 4800 BRAF V600 Mutation Test, the companion diagnostic test for vemurafenib. Using the test to select patients for treatment results in improved outcomes compared to the usual standard of care, dacarbazine, and may also result in improved outcomes compared to ipilimumab, a newly available and favored treatment option, although no direct comparative trial has been conducted. Currently, a trial is underway testing the combination of vemurafenib and ipilimumab (NCT01400451).

**Discussion**

The targeted drug-companion diagnostic test co-development process is a subset of a broader category of “personalized” treatments that use a companion test to select patients most likely to respond, or to avoid treating patients likely to have serious adverse reactions. For example, an evaluation of HLA-B*5701 testing as a companion test for abacavir resulted in the exclusion of test-positive patients from abacavir treatment and the elimination of immunologically-confirmed, serious hypersensitivity reactions (Hughes et al. 2008). In this case, as in other examples, the companion test was developed and evaluated after drug approval. Going forward, as the molecular mechanisms and genetic influences of treatment actions are increasingly understood, companion tests are more likely to be developed in tandem. Targeted drug development, which depends on such an understanding, offers early examples of this process. In recognition, the FDA has provided draft guidance for a parallel and coordinated review and approval process in which both therapeutic treatment and diagnostic test must be approvable, or neither will be approved. As justification, the FDA notes that when use of the treatment depends on the results of the companion test, then there are important concerns regarding the safety and effectiveness of both the therapeutic treatment and the companion diagnostic test.

This Report examined the data supporting the co-review and approval process using vemurafenib and its companion test, the cobas 4800 BRAF V600 Mutation Test, for the treatment of advanced/metastatic melanoma. Based on this example, general caveats to the co-development process require discussion. First, the vemurafenib Phase II and III clinical studies were all of enrichment design, enrolling patients based on a positive result from the companion test. The feasibility of this trial design relies on the knowledge from targeted drug design and from preclinical studies that melanoma tumors with a V600 mutation are likely to respond to...
vemurafenib, whereas tumors without a V600 mutation are unlikely to respond. As a result, smaller, more efficient trials can be conducted to determine drug efficacy. The trade-off, however, is the loss of information regarding the effect of vemurafenib on V600 wild-type melanoma tumors. Although preclinical data suggested that vemurafenib may potentiate tumor growth in V600 wild-type tumors, the comparable effect in patients remains unknown. Thus, the co-development process results in a narrow indication for treatment.

A limitation of the vemurafenib clinical studies was the absence of data on tumor tissue heterogeneity or homogeneity for the $BRAF^{V600E}$ mutation. The cobas 4800 BRAF V600 Mutation Test provides qualitative mutation detection using extracted DNA from a melanoma tissue specimen. Although it is highly sensitive for samples with low percent mutation in studies of analytic validity, clinical trials did not evaluate percent mutation within tumors, i.e., tumor heterogeneity, and correlate that result with response/nonresponse, or degree of response. Studies published by other groups suggest that $BRAF$ mutations are early events in the development of melanocytic tumors, but alone are insufficient to cause primary tumor formation and progression. Tissue from primary tumors positive for $BRAF$ mutations appears to be heterogeneous at a cellular level, but related metastases are consistently $BRAF$ positive if the primary tested positive. Immunohistochemistry studies suggest that metastases are homogenous in terms of mutated protein reactivity, but intensity of staining can be variable, and a more quantitative correlation of $BRAF$ mutation with treatment outcome may be useful. Although this information is preliminary, it is conceivable that improved companion tests or tests that enhance the information provided by the basic companion test may be developed; whether the co-development review process allows for such development is unclear.

In recent years, molecular tests have been carefully evaluated by groups like the EGAPP Working Group and the Agency for Healthcare Research and Quality’s various evidence-based assessment programs. This is because FDA review of diagnostic tests does not usually encompass clinical utility and, in fact, most molecular tests are laboratory-developed tests not submitted to FDA for review.

The companion test development process results in the selection of patient groups that are either highly likely or highly unlikely to respond, seemingly a special case of a predictive test. And while targeted drug development does represent a new era of structural drug design that is more likely to elicit strong and predictable responses, general considerations of test evaluation are not unique. In general, predictive tests attempt to separate a patient population into those with a higher versus a lower likelihood of response to a treatment. Often the separation of groups is insufficient for clinical utility for individual patients, although separation of groups may be statistically significant (clinical validity). Targeted drugs increase the likelihood of response such that clinical utility is more often shown.

This Report specifically asked whether the companion diagnostic co-review and approval process generated sufficient evidence of clinical utility in the example reviewed, such that additional evaluation would not be necessary for future companion tests approved via this process.

The cobas 4800 BRAF V600 Mutation Test was developed sufficiently early in the overall drug-test validation process such that a finalized version of the test was used in both Phase II and key Phase III clinical studies, the latter of which resulted in the clinical data submitted to the FDA. The companion test was co-submitted as a Class III device, requiring a higher level of support data. This was likely required because of potentially higher risk to the patient based on test results than for other types of laboratory tests that are classified as Class I or II devices. Extensive data support analytic validity, the technical performance of the test.

The results of the Phase III trial, supported by the results of the earlier trials, support the clinical validity and clinical utility of the cobas 4800 BRAF V600 Mutation Test, the companion diagnostic test for vemurafenib. Using the test to select patients for treatment results in improved outcomes compared to the usual standard of care, dacarbazine. In addition, comparison of these results with the trial results of the recently approved ipilimumab, suggests that treatment with vemurafenib results in improved outcomes compared to ipilimumab. Ipilimumab is notable as the first therapy to
BRAF Gene Mutation Testing to Select Patients with Melanoma for Treatment with BRAF Kinase Inhibitors

show a survival advantage in a Phase III trial for patients with advanced melanoma, and while vemurafenib was in clinical trials, may have become the new treatment standard for late-stage disease and thus is an important comparator. Currently, a trial is underway testing the combination of vemurafenib and ipilimumab (ClinicalTrials.gov Identifier NCT01400431).

Some important limitations and gaps in knowledge were identified:

- As noted, the vemurafenib companion test identifies some melanoma tumors carrying BRAF V600 mutations other than V600E as positive. There are reports of patients with melanoma tumors positive for V600 mutations other than V600E responding strongly to vemurafenib (Rubinstein et al. 2010), thus such reactivity may prove beneficial. Follow-up on vemurafenib response in this patient population will be important.

- Use of commercially available, non-FDA-approved laboratory developed tests for the BRAF V600E mutations to select patients for vemurafenib treatment is not straightforwardly addressed by the FDA guidance on in vitro companion diagnostics or by other in vitro diagnostic or clinical laboratory regulations. It is possible that several different tests could be used for this purpose; impact on treatment outcomes is unknown.

- Knowledge of the impact of tumor heterogeneity with respect to the BRAF mutation on vemurafenib treatment outcomes may inform patient selection.

- In general, it should be remembered that the development process, using enrichment trial designs, is an efficient process that provides information regarding patients with the molecular target, but provides no information regarding all other patients, who may have received all other treatment possibilities, and for whom any new available treatment may be attractive.

Within the context of the vemurafenib example, FDA approval of the drug and companion test does not constitute the entire story regarding the treatment of melanoma. Despite impressive response rates in the Phase I trial, the duration of response was limited to 2 to more than 18 months, suggesting the development of resistance to vemurafenib; in some patients with BRAF V600E, there was no response at all, which was interpreted as primary resistance. Investigations of the mechanisms of resistance have reported evidence of different molecular mechanisms potentially responsible for resistance in different patients (Johannessen et al. 2010; Nazarian et al. 2010), including the already discussed example of tumors positive for both BRAF V600E and for RAS mutations (see Background, Developing Targeted Treatment: BRAF Inhibitors for Melanoma, Preclinical Development). It is likely that combined inhibition of BRAF and other key molecular targets, and the use of different combinations in different patients, will be needed in the future. For example, a clinical trial is already underway combining treatment with vemurafenib and a MEK inhibitor in patients who have already been treated with vemurafenib (NCT01271803). Ernstoff summarizes the concept by stating, “Melanoma, like other cancers, is a collection of heterogeneous tumors that are differentiated by means of molecular markers, and each molecularly defined subgroup will probably have a different treatment algorithm” (Ernstoff 2011).

Vemurafenib is not the only BRAF inhibitor that has been tested in Phase II or III clinical trials; in a Phase I/II clinical trial of dabrafenib, median progression-free survival was 8.5 months with 70% of 112 patients achieving a partial response (Kefford et al. 2010). Interestingly, in 10 patients with asymptomatic brain metastases, 8 had at least a partial response (5 had a complete response); no progressive brain disease was seen (Long et al. 2010). This suggests activity against brain metastases, however, this is not being examined in the ongoing Phase III clinical trial for patients with BRAF-mutation-positive tumors and advanced melanoma (ClinicalTrials.gov Identifier NCT01227889), which excludes patients with active brain metastases. Dabrafenib is, however, being tested in a Phase II trial for patients with BRAF mutation-positive melanoma tumors and brain metastases (ClinicalTrials.gov Identifier NCT01269687). Another difference between the two BRAF inhibitors is that dabrafenib is not associated with the adverse effects of photosensitivity, reported frequently with vemurafenib.
Conclusion

We conclude that the FDA process for coordinated review and co-approval of both therapeutic treatment and associated companion diagnostic test ensured sufficient validation of the companion diagnostic in the case of vemurafenib and its companion test, the cobas 4800 BRAF V600 Mutation Test. Important limitations and knowledge gaps specific to the example, and to the co-development process in general were identified. We further conclude that review of the final version of the FDA guidance document on in vitro companion diagnostics, as well as additional specific examples, will be important to determine whether the co-development process will be sufficient to ensure sufficient evidence of clinical utility for all companion diagnostics approved via this regulatory review pathway.
References


## Appendix

### Table A. Biochemical IC\textsubscript{50} Determinations of the Inhibitory Activity of PLX4032 versus a Panel of Kinases (Bollag et al. 2010)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Kinase Product Function*</th>
<th>IC\textsubscript{50} nM**</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BRAF</strong> V600E</td>
<td>Mutated version of target kinase.</td>
<td>31</td>
</tr>
<tr>
<td><strong>BRAF</strong> WT</td>
<td>Wild-type version of target kinase.</td>
<td>100</td>
</tr>
<tr>
<td>CRAF</td>
<td>Similar in gene sequence to BRAF; part of the Ras-dependent MAPK/ERK mitogenic signaling pathway from receptors to the nucleus.</td>
<td>48</td>
</tr>
<tr>
<td>SRMS</td>
<td>May be involved in proliferation or differentiation of keratinocytes in the skin.</td>
<td>18</td>
</tr>
<tr>
<td><strong>BRAF</strong> V600E (Not re-assayed at 10μM ATP)</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>TNK2</td>
<td>Nonreceptor tyrosine kinase that regulates the activity of a number of proteins critical for cell survival, cell growth, and proliferation.</td>
<td>19</td>
</tr>
<tr>
<td>MAP4K5 (KHS)</td>
<td>May play a role in the response to environmental stress. Ubiquitously expressed in all tissues examined; high levels in ovary, testis, and prostate.</td>
<td>51</td>
</tr>
<tr>
<td>FGR</td>
<td>The protein localizes to plasma membrane ruffles, and functions as a negative regulator of cell migration and adhesion.</td>
<td>63</td>
</tr>
<tr>
<td>LCK</td>
<td>Plays an essential role for the selection and maturation of developing T-cells in the thymus and in mature T-cell function. Is constitutively associated with the cytoplasmic portions of the CD4 and CD8 surface receptors and plays a key role in T-cell antigen receptor-linked signal transduction pathways.</td>
<td>183</td>
</tr>
<tr>
<td>BRK1</td>
<td>Involved in regulation of actin and microtubule organization.</td>
<td>213</td>
</tr>
<tr>
<td>NEK11</td>
<td>Possible role in S-phase checkpoint mechanism.</td>
<td>317</td>
</tr>
<tr>
<td>BLK</td>
<td>Modulator of beta-cells function; Defects in BLK are a cause of maturity-onset diabetes of the young type 11 (MODY11).</td>
<td>547</td>
</tr>
<tr>
<td>LYN</td>
<td>Down regulates expression of stem cell growth factor receptor (KIT); acts as an effector of erythropoietin receptor in controlling KIT expression and may play a central role in erythroid differentiation.</td>
<td>599</td>
</tr>
<tr>
<td>YES1</td>
<td>?</td>
<td>604</td>
</tr>
<tr>
<td>WNK3</td>
<td>?</td>
<td>877</td>
</tr>
<tr>
<td>MNK2</td>
<td>May play a role in the response to environmental stress and cytokines. Appears to play a role in regulating transcription.</td>
<td>1,717</td>
</tr>
<tr>
<td>FRK (PTK5)</td>
<td>Positively regulates PTEN protein stability; may function as a tumor suppressor.</td>
<td>1,884</td>
</tr>
<tr>
<td>CSK</td>
<td>?</td>
<td>2,339</td>
</tr>
<tr>
<td>SRC</td>
<td>Nonreceptor protein tyrosine kinase that plays pivotal roles in numerous cellular processes such as proliferation, migration, and transformation.</td>
<td>2,389</td>
</tr>
</tbody>
</table>

* From UniProtKB database at http://www.uniprot.org/uniprot/
** All RAF enzymes and SRMS were assayed at an ATP concentration of 100 μM, while all other kinases were assayed at an ATP concentration of 10 μM, resulting in lower IC\textsubscript{50} values.
Validation of the cobas® 4800 BRAF V600 Mutation Test

Information regarding the cobas® 4800 BRAF V600 Mutation Test validation is available as follows: The approval letter, label (kit insert) and summary of safety and effectiveness for the test, released by the Office of In Vitro Diagnostics at the FDA, are available at http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfTopic/pma/pma.cfm?num=P110020. The test kit insert was also released online by Roche, at http://1roche.mylabonline.com/rs/roche/images/BRAF_US-IVDpackage_insert.pdf. Information from these sources is briefly summarized.

The cobas® 4800 BRAF V600 Mutation Test is a real-time polymerase chain reaction (PCR) test intended for the qualitative detection of the BRAF^{V600E} mutation specifically in DNA that has been extracted from formalin-fixed, paraffin-embedded human melanoma tissue. The test kit includes a DNA sample preparation kit for manual isolation of genomic DNA from formalin-fixed, paraffin-embedded (FFPE) tumor tissue samples, the traditional preservation method of biopsy or surgical samples. The test has not been validated for any other type of sample preservation, or any type of tissue or tumor other than melanoma. The kit also includes all necessary reagents for conducting real-time PCR testing, automated on the cobas 4800 instrument, to determine BRAF V600 mutation status.

The PCR reaction uses primers to selectively define and amplify a 116-base pair sequence of human genomic DNA that contains the BRAF codon 600 site in exon 15 of the gene. Separate and differentially labeled wild-type and mutation-specific probes bind to their amplified target sequences and are detected using dedicated optical channels for each probe label.

The test contains different types of quality control. First, the reaction reagents are structured to destroy possible contaminating amplicons (e.g., from prior reactions in the instrument) before the initial amplification of the current sample, without affecting the current PCR reaction. In addition, the test kit contains test mutant and wild-type controls for each run, both of which must return correct results within specified signal ranges for the run to be valid. Finally, BRAF^{WT} alleles from extracted test specimens serve as internal full process controls.

Analytical sensitivity was tested as follows:

- **Testing limit of blank:** Samples with no template and 100% BRAF wild type DNA were evaluated to ensure that a blank sample or a sample with only wild-type DNA would not generate a false analytic signal. All results were as expected.
- **Testing limit of detection:** Replicate measurements were performed on dilution samples that contained various amounts of genomic DNA and various percentages of the V600E mutant sequence, which bracketed the expected analytical sensitivity of the test. The results support a detection limit of 5% V600E alleles in a background of 95% wild-type alleles.
- **Using specimen blends:** DNA isolated from BRAF V600E mutant specimens was blended with DNA from wild-type specimens to achieve mutation levels of 3–10% in various samples. Results: correct calls were consistent at mutation levels of 5% or greater.
- **Using FFPE tumor samples with known mutation levels:** 48 sample from each of 3 specimens at mutation levels of 5–12%. Results: correct calls were consistent at mutation levels of 5% or greater using the required DNA starting amount of 125ng/25µL.
- **Using cell line blends:** DNA from a mutant melanoma cell line was blended with DNA from a wild-type melanoma cell line to achieve a 5% mutation level. The standard sample (125ng/25µL) and dilutions down to 0.5ng/25µL were tested in 20 replicates of each. Results: 95% of replicates resulted in mutation detected at 3.9 ng/25µL, representing a 1:32 dilution of the recommended DNA starting amount.
- **Using simple dilutions of FFPE sample DNA:** Various samples were serially diluted from 250 ng to 31.5 ng/25µL and tested with 2 test kit lot numbers. Results: expected calls were obtained for all samples and dilutions.
- **Determining minimal tumor content:** 33 FFPE specimens with tumor content ranging from 5–50% were tested without macro-dissection. Results: correct calls were obtained when tumor content was 15% or greater. **Macro-dissection for specimens with tumor content less than 50% is required in the kit insert.**

Analytic specificity was determined through an analysis of the sequences of the primers and probes and their alignment. A demonstra-
tion of the alignment of the primers with the 
BRAF gene and with other genes in the RAF family showed the specificity of the primers for the BRAF sequence. In addition, a sequence homology search to predict the specificity of the primers confirmed BRAF specificity.

Cross-reactivity was evaluated by testing the following specimen types:

- FFPE tumor specimens with BRAF non-V600E mutations: 14 specimens with V600D, V600E2, V600R, or V600K mutations were tested in triplicate. Results: only the V600R mutation showed no cross-reactivity (although it was only tested at a low percent mutation level). Cross-reactivity with other mutations depended on the mutation level present in the sample. The kit insert states that the test does not reliably detect non-V600E mutations.

- Plasmids containing BRAF non-V600E mutant DNA at mutation levels ranging from 5 to 75% in a background of wild-type plasmid: mutations tested were D594G, G596R, K601E, L597Q, L597S, V600D, V600E2, V600K, and V600R. Results: Cross-reactivity was seen only for the BRAF V600D plasmid at ≥10% mutation, BRAF V600K plasmid at ≥35% mutation, and BRAF V600E2 plasmid at ≥65% mutation.

- Plasmids containing BRAF homologs: plasmids containing the BRAF pseudogene, ARAF, or RAF1 and wild-type plasmid were tested alone and mixed with 5% BRAF V600E mutant sample. Results: none of the plasmids alone were detected, whereas all of the mixtures were called as mutation positive.

- Skin-related microorganisms: 2 Staphylococcus and 4 Corynebacterium species were each added separately to a wild-type melanoma FFPE tumor sample. Results: none of the test samples were positive for mutation.

Interference from triglycerides, hemoglobin, and necrotic tissue (10-95%, tested in 27 mutation-positive or wild-type specimens) was absent. Melanin is a known inhibitor of PCR reactions. The impact of high concentrations of endogenous melanin was evaluated in 41 FFPE tumor samples selected to represent different levels of pigmentation. Three of the 41 specimens produced “invalid” results and were diluted 2-, 4-, and 8-fold from the starting 125ng/25µL sample. A 2-fold dilution (and all other dilutions) of each sample resulted in a correct call, and is the recommended protocol for invalid sample results. Two highly pigmented BRAF wild-type samples resulted in false-positive calls.

Test reproducibility was evaluated across 5 external testing sites (2 operators per site), 5 test kit lot numbers, and 5 non-consecutive testing days using a panel of 8 samples prepared from various pigmented and non-pigmented tumor samples with ranges of percent tumor content and percent mutant alleles, including one sample near the limit of detection. Ninety-two of 94 test runs were valid (98%). In 92 runs, 2 of 1,442 samples tested gave invalid results (0.14%); all valid test results were correct calls except for the limit of detection sample (90% correct calls). The coefficient of variance was 3% or less for all samples and controls.

Test Repeatability was evaluated internally on 5 different FFPE tumor samples tested on four different days by two operators, using two different test kit lot numbers and two cobas® 4800 instruments. Samples included two with tumor content less than 35% and two with mutation percentages less than 12%. Thirty-two replicates were evaluated for each sample. Average correct calls across all samples was 99%.

Additional studies tested the robustness of the PCR conditions, the ability of the reagents and the procedure to prevent cross-contamination, reagent stability, and overall kit stability including during shipping. Reasonable limits for laboratory use have been demonstrated. Specimen stability was also tested under a variety of conditions. Extracted DNA is stable for at least 61 days stored at -20°C or at 2–8°C, and for at least 8 days stored at 52°C. FFPE specimens are stable at least 9 months at room temperature; 5µm slide-mounted sections from FFPE specimens are stable for at least 91 days at 32°C.

Reference