Author’s response to reviews

Title: Comparative evaluation of the new FDA approved THxIDTM-BRAF test with High Resolution Melting and Sanger sequencing

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Author’s response to reviews: see over
Dear Dr Roselyn Remoto,

Please find attached the revised version of our manuscript 8140137311257400 submitted for consideration for publication as an original article in BMC Cancer. We are delighted that you found our article as potentially acceptable.

We thank all the reviewers for their comments. Detailed response to reviewer’s queries has been submitted separately. We added new references as asked by reviewer 1 and 3. We increased the quality of figure 1.

We hope that this version will meet with your approval and look forward to hearing from you.

Sincerely,

Dr Jérôme Solassol
Reviewer 1:

We wish to thank the reviewer for his comments.

Minor Essential Revisions

1- Introduction section: "The average survival of malignant melanoma is only 8 months following diagnosis with a 5-year survival of less than 10% [2]." The authors should cite a more recently published paper, as the survival time has increased within the last years due to the introduction of (1) BRAF inhibitors and (2) ipilimumab.

This point has been now addressed in the revised version, “introduction” section page 3: “Melanoma is expected to be diagnosed in 76,690 persons in the United States, and 9,480 patients will die of the disease in 2013 [1]. As long as the disease stays localized, cutaneous melanoma presents a favourable prognosis. Indeed, the therapeutic coverage of the early-stage melanoma (stage I and II of the American Joint Committee on Cancer - AJCC) is essentially surgical with a cure rate that approaches 90% [2]. However, a substantial minority will develop disseminated disease (stage IV). The prognosis for patients with stage IV melanoma has historically been poor, with median survival less than 1 year and a 5-year overall survival rate of <10% [3]."


We modified the reference.
Reviewer 2:

We wish to thank the reviewer for his comments.

Major Compulsory Revisions

1- “Page 7, Line 9; I think that the aims of this study include a detailed introduction of a new diagnostic platform to detect BRAFV600E and BRAFV600K mutations in melanoma samples. So, the authors need to indicate more detailed experimental protocols. For examples, the principle of this new platform and the differences compared to HRM and Sanger sequencing should be described “

The THxID™-BRAF kit is a real-time PCR test on the ABI 7500 Fast Dx system and is intended to be used as an aid in selecting melanoma patients whose tumors carry the BRAF V600 mutations. The THxID™-BRAF kit allows detection of the V600E and V600K mutations of the BRAF gene from FFPE sections whereas HRM can only detect changes in the melting profile requiring additional Sanger sequencing to precisely identify the BRAF mutation. THxID™-BRAF kit makes use of 2 major processes:

• Nucleic acid isolation from FFPE sections through extraction / purification steps: The paraffin is removed. The sample is lysed then heated to reverse formalin crosslinking. The DNA is bound to a membrane. After washing, concentrated DNA is eluted from the membrane.

• Real time PCR amplification and detection of BRAF gene present in the total nucleic acids using an Amplification Refractory Mutation Specific System (ARMS) PCR technology. ARMS PCR approaches have been widely and successfully used to detect somatic mutation in routine research, including BRAF mutations (Huang T et al., Sensitive detection of BRAF V600E mutation by Amplification Refractory Mutation System (ARMS)-PCR. Biomarkers, 2013; Hamfjord J et al., Wobble enhanced ARMS method for detection of KRAS and BRAF mutations. Diagn Mol Pathol. 2011; Ellison G et al., A comparison of ARMS and DNA sequencing for mutation analysis in clinical biopsy samples. J Exp Clin Cancer Res. 2010; Machnicki MM et al., ARMS-PCR for detection of BRAF V600E hotspot mutation in comparison with Real-Time PCR-based techniques. Acta Biochim Pol. 2013). One of the advantages of ARMS-PCR is that the assay is designed to amplify a relative larger common fragment of DNA that flanks the mutation site in all samples regardless of their mutation status. This common fragment conveniently serves as an internal control for template DNA quality as well as potential PCR inhibition. The mutant or wild-type specific PCR amplifications take place in the same reaction tube, thereby allowing the mutant or wild-type
specific PCR primers to compete for binding to very limited templates. In the PCR reaction, primers specific for the BRAF gene allow the amplification of a non-polymorphic gene area, which is used as an internal control. The primers specific for the mutations V600E and V600K allow the amplification of mutated fragments leading to the identification of BRAF mutations. Target specific probes bind instantaneously to the newly synthesized complementary DNA. In the THxID™-BRAF kit, 2 different probes labeled with 2 different dyes allow the simultaneous detection of the BRAF internal control and a BRAF mutation. Kinetic analysis of the fluorescent signals and delta Ct (Crossing threshold) calculation reveal the presence of potential BRAF mutations.

We have now detailed more precisely the principle of the THxID™-BRAF kit in the revised manuscript in the “Methods” section page 8: “The principle of the THxID™-BRAF assay kit is based on an ARMS-PCR approach [11]. In the PCR reaction, primers specific for the BRAF gene allow the amplification of a non-polymorphic gene area, which is used as an internal control. The primers specific for the mutations V600E and V600K allow the amplification of mutated fragments leading to the identification of BRAF mutations whereas HRM can only detect changes in the melting profile requiring additional Sanger sequencing to precisely identify the BRAF mutation. In THxID™-BRAF kit, 2 different probes labeled with 2 different dyes allow the simultaneous detection of the BRAF internal control and a BRAF mutation. Kinetic analysis of the fluorescent signals and delta Ct (Crossing threshold) calculation reveal the presence of potential BRAF mutations. The qualitative results (wild-type, mutated V600E and/or V600K, invalid) are supplied as a report.”

We also modified the “Discussion” section page 12: “THxID™-BRAF used an ARMS based PCR technology. ARMS PCR approaches have been widely and successfully used to detect somatic mutation in routine research, notably BRAF mutations [16, 29, 30]. One of the advantages of ARMS-PCR is that the assay is designed to amplify a relative larger common fragment of DNA that flanks the mutation site in all samples regardless of their mutation status. In our PCR reaction, primers specific for the BRAF gene allow the amplification of a non-polymorphic gene area, which was used as an internal control to check for template DNA quality as well as potential PCR inhibition. The mutant or wild-type specific PCR amplifications take place in the same reaction tube, thereby allowing the mutant or wild-type specific PCR primers to compete for binding to very limited templates.”
The THxID™-BRAF software interprets the results automatically and highlights the presence of valid or invalid results in the generated report. The 2 possible outcomes for Positive and Negative Controls are "valid" or "invalid". If one or more controls are invalid, the results of the clinical specimen obtained in the run are not reported. In such case, the complete run must be repeated using frozen sample eluates, frozen Negative Control eluate and either frozen Positive Control if available or a new preparation.

The result validity of clinical specimens is determined first by the internal control Ct values that should fall within pre-specified limits. If a specific amplification is detected for the mutant target, the result of each reaction (V600E or V600K) is based on the delta Ct value (Ct mutant– Ct IC): If the delta Ct value is below a threshold value then a V600E or V600K BRAF mutation is present; If the delta Ct value is above a threshold value then no V600E or V600K BRAF mutation is present or it is below the limit of detection; If no amplification is detected for the mutant targets (V600E and V600K not detected), the sample will be characterized as BRAF mutation-negative. Results cannot be validated if the control values are not valid. A result is invalid if one of the delta Ct or internal control Ct values falls outside the expected limits.

An invalid result of clinical specimen in a valid run can have several explanations:
- Too much DNA in the reaction: the eluate has to be diluted 1:4 in Buffer.
- Too low DNA in the reaction and/or PCR inhibition. If the tested sample has been characterized as containing no or a low level of melanin (≤10%) by the pathologist, repeat the test, starting from the eluate. If the result is still invalid, repeat the test for the invalid sample, starting from the extraction using higher tissue area. If the tested sample has been characterized as containing a medium to high level of melanin (> 10%) by the pathologist, the eluate has to be diluted 1:4 in Buffer ATE before amplification by adding 10 µL of eluate to 30 µL of Buffer ATE. If the result is still invalid, repeat the test starting from the extraction using higher tissue area.

Sanger sequencing and HRM test results were considered as invalid because of amplification failure. The failure of fragment amplification could probably be due to the high degradation of FFPE-used material, as largely reported previously (Srinivasan M, et al. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol. 2002).
This point has been addressed in the revised version of the manuscript in a new paragraph of the “Methods” section page 8: “Validation or invalidation test definitions: The THxID™-BRAF software interprets the results automatically and highlights the presence of valid or invalid results in the generated report. The 2 possible outcomes for Positive and Negative Controls are "valid" or "invalid". If one or more controls are invalid, the results of the clinical specimen obtained in the run are not reported. In such case, the complete run must be repeated using frozen sample eluates, frozen Negative Control eluate and either frozen Positive Control if available or a new preparation. The result validity of clinical specimens is determined by the internal control Ct values that should fall within pre-specified limits, following the manufacture’s recommendations. A result is considered as invalid if one of the delta Ct or internal control Ct values falls outside the expected limits. In our study, Sanger sequencing and HRM test results were considered as invalid when DNA could not be amplified. The failure of fragment amplification could probably be due to the high degradation of FFPE-used material, as largely reported previously [12].”

3- “Page 13, line 22; The authors said that Sanger sequencing has low analytic sensitivity. To support their conclusion, the authors need to perform HRM, Sanger sequencing and THxID™-BRAF kit to detect BRAFV600E in DNA mixtures with mutant DNA percentages ranging from 0% to 100% (Clin Endocrinol (Oxf). 2009 Jan;70(1):139-44.)."

previously explained in the conclusion of our manuscript, we are confident that Sanger sequencing has pretty “low analytic sensitivity” even if we do not have assessed that. THxIDTM-BRAF kit technical validation including test sensitivity, cross-reactivity, interfering substances determination, repeatability (within-laboratory precision), reproducibility (between-laboratory precision) has been previously performed by Biomerieux US for FDA validation. All results are detailed in the instruction’s recommendations. Briefly, the Limit of Detection (LoD) assay was assessed and determined by Probit analysis (the calculation was based on the assumption that the starting material extracted from the mutant specimens contained 100% mutant DNA). The data support a claimed LoD of 5% mutant DNA in a background of wild-type DNA for V600E and V600K positive FFPE skin and lymph node specimens across the DNA input range. In addition, the potential background amplification was assessed on DNA extracted from 3 cell lines (wild-type, V600E homozygous, V600K heterozygous) at high DNA input (350 ng/µL). The THxID™-BRAF assay did not show any background amplification in all tested conditions.

In our study, we used a commercialised BRAF genotyping already FDA approved kit in order to evaluate its clinical relevance for BRAF mutation detection in routine and compared our results with standardised approaches (Sanger sequencing and HRM). We believe that testing the sensitivity in DNA mixtures with mutant DNA percentages ranging from 0% to 100% would not be need here and would be more available for ‘home-made’ new procedures. However, for a better clarity of the manuscript, we indicated page 8 that THxIDTM-BRAF kit technical validation has been performed previously: “The qualitative results (wild-type, mutated V600E and/or V600K, invalid) are supplied as a report. THxIDTM-BRAF kit technical validation has been performed previously by Biomerieux US and is reported in manufacture’s recommendations.”

Minor Essential Revisions

“Figure 1; Letters on Fig 1A and 1B are not clear. The resolution should be Improved”

This point has been now addressed in the revised version.
Reviewer 3:

We wish to thank the reviewer for his comments.

Minor Essential Revisions

“Since the major theme of the research is to validate and compare the efficiency of the THxID™-BRAF Kit, the authors could have discussed the average time taken to analyze "n" number of samples for detection of the specific mutations in the samples.”

This point has been now addressed in the revised version in the “conclusion” section page 15: “In addition, THxID™-BRAF is a simple and fast procedure that does not entail any special equipment other than a thermocycler. The estimated duration time for this protocol is less than 24 hours (from the reception of the sample to the delivery of the final report to clinician). Since the response time for tests of BRAF status is a major issue, this procedure could also offer new opportunities to significantly enhance sample throughput, decrease turnaround time, and propose an interesting alternative solution in “urgent” cases.”