A multisite blinded study for the detection of \textit{BRAF} mutations in formalin-fixed, paraffin-embedded malignant melanoma

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Melanoma patients with \textit{BRAF} mutations respond to treatment with vemurafenib, thus creating a need for accurate testing of \textit{BRAF} mutation status. We carried out a blinded study to evaluate various \textit{BRAF} mutation testing methodologies in the clinical setting. Formalin-fixed, paraffin-embedded melanoma samples were macrodissected before screening for mutations using Sanger sequencing, single-strand conformation analysis (SSCA), high resolution melting analysis (HRM) and competitive allele-specific TaqMan \textsuperscript{\textregistered} PCR (CAST-PCR). Concordance of 100\% was observed between the Sanger sequencing, SSCA and HRM techniques. CAST-PCR gave rapid and accurate results for the common V600E and V600K mutations, however additional assays are required to detect rarer \textit{BRAF} mutation types found in 3–4\% of melanomas. HRM and SSCA followed by Sanger sequencing are effective two-step strategies for the detection of \textit{BRAF} mutations in the clinical setting. CAST-PCR was useful for samples with low tumour purity and may also be a cost-effective and robust method for routine diagnostics.

\textbf{Mutations in the \textit{BRAF} oncogene are found in approximately 50\% of all sun-exposed melanomas\textsuperscript{1–5}. The majority of mutations (70–80\%) comprise a single base substitution in codon 600, identified as c.1799 T \rightarrow A \textit{p.Val600Glu} and commonly referred to as V600E. Of the remaining \textit{BRAF} mutations in melanoma, by far the most common involves the mutation of two adjacent nucleotides and is identified as c.1798_1799delinsAA \textit{p.Val600Lys}, or V600K\textsuperscript{3,5–8}. Rarer mutations include c.1798_1799delinsAG \textit{p.Val600Arg} (V600R), c.1801A \rightarrow G \textit{p.Lys601Glu} (K601E) and c.1799_1800delinsAA \textit{p.Val600Glu} (V600E2).} \

\textit{BRAF} mutation results in hyperactivation of the MAPK signalling pathway, causing deregulation of cell proliferation and oncogenesis without the requirement for Ras activation\textsuperscript{1,9}. Braf is the most potent of the Raf proteins to activate the downstream signalling cascade, hence mutant Braf was identified as a novel target for kinase inhibitors such as vemurafenib (PLX4032/RG7204)\textsuperscript{6,10,11} and dabrafenib\textsuperscript{12}. \

In a recent phase 3 trial, treatment with vemurafenib was associated with improved survival of metastatic melanoma patients with the \textit{BRAF} 600E mutation\textsuperscript{13}. As a result of these clinical findings, vemurafenib was approved by the US Food and Drug Administration (FDA) for the treatment of advanced stage melanoma harbouring the V600E mutation. Promising data has also been reported with the Braf inhibitor dabrafenib\textsuperscript{12,14}. Accurate determination of the \textit{BRAF} status of melanomas is therefore crucial in deciding upon the use of Braf inhibitors in individual patients. For this purpose a companion diagnostic kit, the Cobas 4800 BRAF V600 mutation test (Roche Diagnostics) was also approved by the FDA. Although this test screens for the V600E mutation, it also shows some cross reactivity for the V600K mutation\textsuperscript{15}. There is some evidence to suggest that patients with the V600K mutation and other rarer codon 600 and 601 mutations also respond to Braf or MEK inhibitors\textsuperscript{13,16–19}. Hence the identification of these and other non-V600E mutant cases is critical to allow stratification of patients for possible treatment.
Table 1 | BRAF mutation detection in melanoma samples using four different methods

| Sample | HRM | SEQ | SSCA | CAST | Mutation 1 | AA change |
|--------|-----|-----|------|------|------------|-----------|
| P01    |     |     |      |      | wildtype   |           |
| P04    |     |     |      |      | c.1798_1799delinsAA, p.Val600Lys V600K |
| P05    |     |     |      |      | wildtype   |           |
| P06    |     |     |      |      | c.1798_1799delinsAA, p.Val600Lys V600K |
| P08    |     |     |      |      | c.1799T>A, p.Val600Glu V600E |
| P09    |     |     |      |      | wildtype   |           |
| P11    |     |     |      |      | c.1798_1799delinsAA, p.Val600Lys V600K |
| P12    |     |     |      |      | c.1799T>A, p.Val600Glu V600E |
| P13    |     |     |      |      | c.1799T>A, p.Val600Glu V600E |
| P14    |     |     |      |      | c.1799T>A, p.Val600Glu V600E |
| P15    |     |     |      |      | wildtype   |           |
| P17    |     |     |      |      | c.1801A>G, p.Lys601Glu K601E |
| P19    |     |     |      |      | wildtype   |           |
| P20    |     |     |      |      | c.1799T>A, p.Val600Glu V600E |
| P21    |     |     |      |      | c.1799_1800delinsAA, p.Val600Glu V600E2 |
| P22    |     |     |      |      | c.1799_1800delinsAA, p.Val600Glu V600E2 |
| P23    |     |     |      |      | c.1799_1800delinsAA, p.Val600Glu V600E2 |
| P24    |     |     |      |      | wildtype   |           |
| P25    |     |     |      |      | c.1798_1799delinsAA, p.Val600Glu V600K |
| P26    |     |     |      |      | c.1798_1799delinsAA, p.Val600Glu V600E |
| P27    |     |     |      |      | wildtype   |           |
| P28    |     |     |      |      | c.1799T>A, p.Val600Glu V600E |
| P29    |     |     |      |      | c.1799T>A, p.Val600Glu V600E |
| P30    |     |     |      |      | wildtype   |           |
| P31    |     |     |      |      | c.1799T>A, p.Val600Glu V600E |
| P32    |     |     |      |      | wildtype   |           |
| P33    |     |     |      |      | c.1799T>A, p.Val600Glu V600E |
| P34    |     |     |      |      | c.1798_1799delinsAA, p.Val600Glu V600K |
| P35    |     |     |      |      | c.1801A>G, p.Lys601Glu K601E |
| P37    |     |     |      |      | c.1798_1799delinsAA, p.Val600Glu V600K |
| P38    |     |     |      |      | wildtype   |           |
| P39    |     |     |      |      | c.1798_1799delinsAA, p.Val600Glu V600K |
| P40    |     |     |      |      | c.1798_1799delinsAA, p.Val600Glu V600K |
| P41    |     |     |      |      | wildtype   |           |
| P42    |     |     |      |      | c.1798_1799delinsAA, p.Val600Glu V600K |
| P43    |     |     |      |      | wildtype   |           |
| P44    |     |     |      |      | c.1799T>A, p.Val600Glu V600E |
| P45    |     |     |      |      | wildtype   |           |
| P46    |     |     |      |      | c.1799_1801delinsAGG, p.Val600_Lys601delinsGluGlu V600E K601E |
| P47    |     |     |      |      | c.1801A>G, p.Lys601Glu K601E |
| P48    |     |     |      |      | c.1798_1799delinsAA, p.Val600Glu V600K |
| P49    |     |     |      |      | c.1799T>A, p.Val600Glu V600E |
| P50    |     |     |      |      | c.1798_1799delinsAA, p.Val600Glu V600K |
| M01    |     |     |      |      | wildtype   |           |
| M02    |     |     |      |      | wildtype   |           |
| M03    |     |     |      |      | c.1798_1799delinsAG, p.Val600Arg V600R |
| M04    |     |     |      |      | wildtype   |           |
| M05    |     |     |      |      | wildtype   |           |
| M06    |     |     |      |      | wildtype   |           |
| M07    |     |     |      |      | wildtype   |           |
| M08    |     |     |      |      | wildtype   |           |
| M09    |     |     |      |      | wildtype   |           |
We and others have reported the frequency of the V600K mutation may be as high as one third of all BRAF mutations in melanoma. Indeed, our earlier study of 183 consecutive cases of metastatic melanoma found the ratio of V600K to V600E mutation was almost 1:2.5. Because some of the assays commonly used in the clinical setting may underestimate the frequency of V600K mutation, the aim of the current study was to evaluate four different platforms for the detection of BRAF mutations and to assess the sensitivity and specificity of each platform. We report here the results of a two institute, blinded study on 93 melanoma samples using the mutation detection methods of Sanger dideoxy sequencing, single strand conformation analysis (SSCA), high resolution melting analysis (HRM) and competitive allele-specific TaqMan (CAST)-PCR (Life Technologies).

| Sample | HRM | SEQ | SSCA | CAST | Mutation | AA change |
|--------|-----|-----|------|------|----------|------------|
| M10    | ●   | ●   | ●    | ●    | c.1799T>A, p.Val600Glu | V600E      |
| M11    | ●   | ●   | ●    | ●    | c.1799T>A, p.Val600Glu | V600E      |
| M12    | ○   | ○   | ○    | ○    | wildtype |            |
| M13    | ●   | ●   | NA   | NA   | c.1798_1799delinsAA, p.Val600Lys | V600K      |
| M14    | ○   | ○   | ○    | ○    | wildtype |            |
| M15    | ○   | ○   | ○    | ○    | wildtype |            |
| M16    | ○   | ○   | ○    | ○    | wildtype |            |
| M17    | ●   | ●   | ●    | ●    | c.1799T>A, p.Val600Glu | V600E      |
| M18    | ○   | ○   | ○    | ○    | wildtype |            |
| M19    | ○   | ○   | ○    | ○    | wildtype |            |
| M20    | ○   | ○   | ○    | ○    | wildtype |            |
| M21    | ●   | ●   | ●    | ●    | c.1798_1799delinsAA, p.Val600Lys | V600K      |
| M22    | ●   | ●   | ●    | ●    | c.1799T>A, p.Val600Glu | V600E      |
| M23    | ○   | ○   | ○    | ○    | wildtype |            |
| M24    | ○   | ○   | ○    | ○    | wildtype |            |
| M25    | ○   | ○   | ○    | ○    | wildtype |            |
| M26    | ○   | ○   | ○    | ○    | wildtype |            |
| M27    | ○   | ○   | ○    | ○    | wildtype |            |
| M28    | ○   | ○   | ○    | ○    | wildtype |            |
| M29    | ●   | ●   | ●    | ●    | c.1799T>A, p.Val600Glu | V600E      |
| M30    | ●   | ●   | ●    | ●    | wildtype |            |
| M31    | ●   | ●   | ●    | ●    | wildtype |            |
| M32    | ●   | ●   | ●    | ○    | c.1801A>G, p.Lys601Glu | K601E      |
| M33    | ●   | ●   | ●    | ○    | c.1799T>A, p.Val600Glu | V600E      |
| M34    | ○   | ○   | ○    | ●    | wildtype |            |
| M35    | ●   | ●   | ●    | ●    | wildtype |            |
| M36    | ○   | ○   | ○    | ○    | wildtype |            |
| M37    | ●   | ●   | ●    | ●    | wildtype |            |
| M38    | ○   | ○   | ○    | ○    | wildtype |            |
| M39    | ○   | ○   | ○    | ○    | wildtype |            |
| M40    | ●   | ●   | ●    | ●    | c.1798_1799delinsAA, p.Val600Lys | V600K      |
| M41    | ●   | ●   | ●    | ●    | c.1799T>A, p.Val600Glu | V600E      |
| M42    | ●   | ●   | ●    | ●    | wildtype |            |
| M43    | ○   | ○   | ○    | ○    | wildtype |            |
| M44    | ○   | ○   | ○    | ○    | wildtype |            |
| M45    | ○   | ○   | ○    | ○    | wildtype |            |
| M46    | ○   | ○   | ○    | ○    | wildtype |            |
| M47    | ○   | ○   | ○    | ○    | wildtype |            |
| M48    | ○   | ○   | ○    | ○    | wildtype |            |
| M49    | ○   | ○   | ○    | ○    | wildtype |            |
| M50    | ●   | ●   | ●    | ●    | c.1798_1799delinsAA, p.Val600Lys | V600K      |

1The mutant sequence was determined in all cases by bidirectional Sanger sequencing. HRM, high resolution melt analysis; SEQ, Sanger sequencing; SSCA, single strand conformation analysis; CAST, CAST-PCR; AA, amino acid; open circle represents wild type BRAF; closed circle represents mutant BRAF.

Results
The results of mutation testing are summarized in Table 1. Of 93 samples tested, 91 gave results using all four platforms. One sample (M05) could not be adequately sequenced and another (M13) failed analysis with both SSCA and CAST-PCR. Representative examples of data output using the sequencing, SSCA, HRM and CAST-PCR methods are shown in Figure 1 for wildtype BRAF and for the V600E, V600K and K601E mutations. A total of 24 V600E, 18 V600K, 4 K601E, 2 V600E2 and one V600R mutation were detected. An exchange of both amino acids at codons 600 (Valine) and 601 (Lysine) (c.1799_1801delinsAGG, p.Val600_Lys601delinsGluGlu, V600E/K601E) was observed in one sample (P46, Figure 2A).
Concordance between HRM, SSCA and sequencing for the detection of mutations was 100%, although only the sequencing method was capable of identifying the exact mutation. CAST-PCR detected all V600E and V600K mutations, but failed to detect the K601E, V600E2 and V600R cases because probes specific for these mutations were not used in the analysis. Interestingly, the double V600E/K601E mutation (P46) was detected by CAST-PCR (Figure 2D) with the V600E probe. Some cross reactivity was observed by CAST-PCR between the V600K probe and V600E mutations (Figure 1N and 3D), but not vice versa (Figure 1O). In two cases (P33, M33) the threshold dCt value was reached with both probes, with dCt values of 2.77 and 9.45, and 3.52 and 8.98 for the V600E and V600K probes, respectively (Figure 3D). Sequencing, SSCA and HRM results confirmed that both cases were V600E (P33 shown in Figure 3 A–C).

For two samples (P08, P14), CAST-PCR detected a V600E mutation that was not found by sequencing, SSCA or HRM (P08 shown in Figure 4). The amount of mutant allele for these samples was estimated using the Mutation DetectorTM software (Life Technologies, USA) to be just 1.7% and 2.2%, respectively. The dCt values for both samples were confirmed to be < 9.96 in two separate runs (Figures 4D and 4E), thus signalling the presence of a mutation. The CAST-PCR results were validated using limited copy number (LCN)-HRM which identifies low frequency mutations by limiting dilution and enables identification of the mutation by Sanger sequencing20–22. Analysis of sample P14 using LCN-HRM revealed the presence of a V600E mutation at an estimated allele frequency of less than 5% (Figure 5).

**Discussion**
This blinded study to evaluate **BRAF** mutation screening methods was carried out by two laboratories with extensive experience in HRM, SSCA and Sanger sequencing. A concordance of 100% for mutation detection was found with all 3 methods for 91 melanoma samples (Table 1). The only discordance was the identification of one sample (P22) as V600E by HRM but as V600K by CAST-PCR and the SSCA banding pattern. Sanger sequencing confirmed this sample was V600K. In this instance, CAST-PCR was very useful in confirming the exact mutation and it also demonstrated an ability to detect low level mutations which can be difficult to read from Sanger sequencing. Similar to previous results with **KRAS** mutation testing23, the present study demonstrated a high concordance for **BRAF** mutation detection when screening was carried out by experienced laboratories working in a clinical setting.

Although HRM and SSCA are rapid, inexpensive and sensitive screening techniques, the drawback is that they do not identify the exact mutant sequence. CAST-PCR also proved in the present study to be a rapid and sensitive technique for **BRAF** mutation screening.
Figure 2 | Results for sample P46 containing the double mutation c.1799_1801delinsAGG (p.Val600_Lys601delinsGluGlu). This mutation was detected by CAST-PCR with the V600E probe (D), as well as by SSCA (B) and HRM (C).

Figure 3 | Results for sample P33 showing cross reactivity between the V600K probe (orange line) and a V600E mutation (blue) in CAST-PCR (D). In this case the dCt value for the V600K assay was lower than the threshold value of 9.96, however Sanger sequencing (A) clearly showed this to be a V600E mutation. The results for SSCA (B) and HRM (C) also suggest a single V600E mutation.
This method was able to identify samples containing as little as 2% mutant allele (Figure 4), confirming recent findings by Didelot et al. on the high sensitivity of this method. CAST-PCR is therefore ideal for samples with low tumour purity. However, the clinical relevance of very low mutant allele content for the response of melanoma to BRAF inhibitors is currently unknown. Treatment of wild type BRAF melanoma cells with inhibitors of the MAPK pathway may in some circumstances stimulate the growth of these cells. Genotypic heterogeneity within tumours and between primary and metastatic tumours may also give rise to low mutant allele content. Further studies are required to determine whether the treatment of patients with apparently low percentages of mutant allele has an overall positive or negative clinical impact.

The present work on CAST-PCR used assays for the detection of V600E and V600K mutations only, meaning that rarer mutations such as V600E2, V600R and K601E were undetected (Table 1). In a recent study of 1,112 unselected cases, non-V600E and non-V600K mutations were estimated to occur in approximately 3–4% of melanomas. Probes for the rare BRAF mutations are commercially available for CAST-PCR but would add to the cost of screening. The assay cost to screen both the V600E and V600K mutations is approximately $US25 per sample. Widening the screen to include the rarer K601E, V600E2 and V600R mutations would add another $US30 per sample, although this could be carried out as a second step only for the 50–60% of samples with apparently wild type BRAF. A possible limitation of the CAST-PCR method in some circumstances is the requirement for relatively large amounts of DNA given that multiple assays are required. Using the manufacturer’s recommendation of 20 ng of DNA per assay, at least 120 ng of DNA would be required to screen for the V600E, V600K, V600E2, V600R and K601E mutations. However, in the current study we were able to use as little as 5 ng of DNA per reaction, suggesting this may not be a serious drawback for the vast majority of samples.

This study also confirms the use of LCN-HRM for identifying low allele frequency mutations in a sequence agnostic fashion. In turn, this has further validated the CAST-PCR approach for low frequency mutations.

Sanger sequencing is considered by many to be the gold standard for mutation detection and has the advantage over many of the rapid screening methods in being able to identify the exact mutation.

Figure 4 | Results for sample P08 showing wildtype profiles (blue arrows) by sequencing (A), SSCA (B) and HRM (C). Two separate runs of CAST-PCR (D, E) revealed a V600E mutation for this sample, with dCt values in each case of <9.96.

Figure 5 | Two samples (P8 and P14) were positive for the BRAF V600E mutation using CAST-PCR but negative using SSCA, HRM and Sanger sequencing. Shown are representative sequencing traces from one of the positive replicates for each of P8 and P14 using LCN-HRM. Red arrows indicate the BRAF V600E mutation.
sequence. However in the routine clinical setting it suffers from two major limitations. Firstly, macrodissection of the specimen by a pathologist is required to ensure the tumour cell content is at least 25%. Secondly, the time taken to obtain the sequence readout can be up to several days depending on the turnaround times of individual and service laboratories. The perfect concordance observed in the present study between Sanger sequencing and the HRM and SSCA methods is likely due to the routine enrichment of tissue samples with tumour following careful evaluation of H&E sections by the pathologist.

Several other methods not tested here have been used for BRAF mutation screening, including pyrosequencing, competitive amplification of differentially melting amplicons (CADMA)27, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS)28, allele-specific PCR (Taqman)14 and quantitative allele-specific PCR using a heterozygous plasmid containing wild-type and mutant sequences as a calibrator13. Pyrosequencing is a robust methodology but in Australia at least this platform is limited by restricted distribution of the instrumentation. Next generation sequencing platforms including Illumina, Helicos and 454 pyrosequencing have also been used to detect BRAF V600E mutations in melanoma29, but these are inefficient unless multiple loci are screened. Immunohistochemistry with the VE1 antibody (Ventana) was recently shown to be highly specific for the V600E mutation and whilst data remains limited, no cross-reactivity with other BRAF mutation has been reported. If used in isolation, immunohistochemistry with VE1 antibody will miss significant numbers of non-V600E BRAF mutations and at times weak staining and heavy melanin pigment makes interpretation difficult requiring confirmation by molecular methods30,31. A two step screening procedure involving immunohistochemistry followed by pyrosequencing for VE1-negative or uninterpretable cases was proposed by Colomba et al32.

The FDA approved Cobas® 4800 BRAF V600 Mutation Test is based upon the use of allele-specific Taqman probes directed at the V600E mutation (c.1799T > A)15,33,39,40. This test was reported to have a very high sensitivity when the V600E mutant allele content was greater than 10%30,32. However, a limitation of the Cobas test is its ability to detect non-V600E mutations, estimated to account for one quarter of all BRAF mutations in a study of 1,112 cases of melanoma16. Anderson et al. reported the Cobas BRAF V600 assay was able to detect 70% of V600K mutant samples through cross-reaction with the TaqMan V600E probe41. Further studies are needed to determine whether this result can be reproduced in independent laboratories where the Cobas test is run in parallel with other mutation detection methods. It also remains to be determined whether the frequency of cross-reaction with V600K is influenced by the mutant allele content. This is especially important in light of clinical findings that suggest V600K mutant tumours respond to vemurafenib30.

The demonstration of clinical activity for Braf inhibitors has created the need for accurate, robust, rapid and cost-effective BRAF mutation screening assays. The present work has shown that HRM and SSCA are able to meet these criteria in the clinical setting and are thus useful as a first screen prior to confirmation and identification of the mutation by Sanger sequencing. The two major limitations of Sanger sequencing are the need to enrich for tumour cell content using macrodissection and the long turnaround time. CAST-PCR was shown here to be a sensitive, rapid and robust method for BRAF mutation screening and can be cost effective through the use of a two step screening procedure. This would involve an initial screening for the V600E and V600K mutations, followed by screening for the rarer mutations only in those samples showing apparently wildtype status. Although HRM, SSCA or allele-specific real-time PCR followed by Sanger sequencing are used routinely in many laboratories, several other strategies for achieving complete BRAF mutation screening are also likely to be effective and could include two step CAST-PCR, pyrosequencing alone, or immunohistochemistry for V600E followed by Sanger sequencing for non-V600E cases. Ultimately, the choice of method used is also dependent on the clinical importance in identifying the 3–4% of melanoma cases known to harbour non-V600E/non-V600K mutations30,32. As more data emerges on the activity of Braf inhibitors in such patients, it is expected that diagnostic methods capable of detecting these mutations will become standard.

**Methods**

**Melanoma samples.** A total of 43 formalin-fixed and paraffin embedded (FFPE) melanoma samples were obtained from the PathWeld laboratory, Sir Charles Gairdner Hospital, Perth. These comprised a selected subset from a consecutive series that had previously been investigated for BRAF mutation by bidirectional Sanger sequencing and in some cases SSCA1. In addition, all samples were evaluated by CAST-PCR. A further 50 FFPE melanoma samples were obtained from the Melbourne Melanoma Project (http://melbournemelanomaproject.com/) for the study was obtained from the human research ethics committees at the Peter MacCallum Cancer Centre and the Sir Charles Gairdner Hospital. Cases from both sites were enriched for tumour cell content by macrodissection following evaluation of the H&E section by a specialist pathologist. Approximately 1–3 cm² of tissue sections (5 μm thickness) was used to extract DNA from each sample. DNA from the Perth samples was sent to Melbourne for analysis using HRM and sequencing, while samples from Melbourne were sent to Perth for analysis by sequencing, SSCA and CAST-PCR. All testing was carried out blinded to the result obtained from the other laboratory. In the Perth laboratory, testing with SSCA, sequencing and CAST-PCR were carried out blinded to results from the other tests. In the Melbourne laboratory, sequencing was carried out only on samples showing a positive or equivocal result with HRM. Controls were conducted for all assays and included the absence of DNA and samples with known wild type or mutant status.

**Single strand conformation analysis (SSCA).** The primers used for fluorescent SSCA were identical to those used for dideoxy sequencing, but with additional fluorescent HEX dye labelling at the 5’ end (GeneWorks, Adelaide, Australia). PCR conditions were the same as for sequencing. Three μl of fluorescent PCR product was mixed with 6 μl deionized formamide loading buffer and heated to 95°C for 2 min and then loaded onto a 20% polyacrylamide glycerol gel (100 μm thick, 18 cm long) and run in a DNA fragment analyser (GS-2000, Corbett Life Sciences, Australia). After pulsing for 20 sec at 1400 V the wells were rinsed and the gel was run at 1400 V for 90 min in 0.8× TBE buffer at a constant gel temperature of 24°C. ONE-Dscan 1.3 software (Scanalytics, Billerica, MA) was used to analyse the gel run. The mutation status of the samples was determined by comparison of the sample bands to those of wild type and mutant controls run in parallel.

**CAST-PCR.** Samples were analysed by CAST-PCR using the BRAFT_473_mu and BRAFT_473_pm probes for the detection of V600E and V600K mutations, respectively (Life Technologies, USA). Mastermixes were prepared as recommended by the manufacturer and distributed in a 96-well plate. DNA for Perth (20 ng) and Melbourne (5 ng) samples was added to each well and the reaction carried out in a Via™ 7 Real-Time PCR system. A lower amount of DNA was used for the Melbourne samples due to the limited availability of this resource. The PCR conditions comprised an initial denaturation step of 10 minutes at 95°C followed by 15 cycles of 15 sec denaturation at 95°C and one minute extension at 58°C. This was followed by 40 cycles of 15 sec denaturation at 95°C and one minute extension at 60°C. Real time data was collected during the last 40 cycles of amplification and analysed using the Mutation Detection™ software v2.0 (Life Technologies, USA). Samples with a delta(Ct) of less than 9.96 were considered positive for mutation, where delta Ct = Ct mut – Ct ref.

**High resolution melt (HRM) analysis.** PCR and HRM were performed using the LightCycler 480 (Roche Diagnostics). The primer sequences used were 5’-CCTCAGACGTTAAATAGGTGATTTTGG-3’ and 5’-GGATCCGACAGACTTGGACTGTA-3’, giving an amplicon size of 88 bp. The reaction mixture included 1× PCR buffer, 2.5 μM MgCl₂, 200 nM of each primer, 200 μM of dNTPs, 5 μM of SYTO 9 (Invitrogen, Carlsbad, CA, USA), 0.5U of HotStar Taq polymerase (Qiagen), 10 ng DNA and PCR grade water in a total volume of 10 μl. PCR conditions included an activation step of 15 min at 95°C followed by 55 cycles of 95°C for 10 sec, annealing for 10 sec comprising 10 cycles of a touchdown from 65°C to 55°C at 1°C cycle followed by 35 cycles at 55°C, and extension at 72°C for 30 sec.

**Limited copy number – high resolution melt (LCN-HRM) analysis.** To examine whether a low frequency of BRAF V600E mutation was present in two samples (P8, P14) that were positive by CAST-PCR but negative by HRM, SSCA and Sanger sequencing, limited copy number – high resolution melting (LCN-HRM) was performed as previously described16. In brief, DNA from these samples was diluted to enable only a few copies of template to be added to a LCN-HRM reaction. CAST-PCR and HRM were performed on the RotorGene Q (Qiagen) and positive LCN-HRM reactions showing aberrant melting profiles were chosen for Sanger sequencing. The LCN-HRM reaction mixture in a total volume of 20 μl was prepared.
as follows for an estimated 2–4 copies of template: 1.

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Biosystems, Life Technologies, Foster City, CA) under the following conditions: an

Sanger sequencing.

Sanger sequencing (Perth laboratory). Bidirectional Sanger sequencing was performed as described earlier. Briefly, PCR was carried out in a final volume of 25 μl using the forward primer sequence 5'-CTCATACTCTGTCTCCTAGTGA-3' and the reverse primer sequence 5'-GGCAGAAGCTACAGCAGAAGC-3', giving an amplicon size of 224 base pairs. PCR reactions contained 10-50 ng of DNA, 0.5 μM of each primer, 200 μM of dNTPs, 5 μM of SYTO9 and 0.5 U of Hotstar Taq polymerase. PCR conditions included an activation step of 15 min at 95°C followed by 60 cycles of 95°C for 10 sec, annealing for 20 sec comprising 10 cycles of a touchdown from 65°C to 60°C at 0.5°C/cycle followed by 50 cycles at 60°C, and extension at 72°C for 30 sec. Each sample was tested in 66 LCN-HRM replicates.

LCN-HRM products showing aberrant melting profiles were used as templates for Sanger sequencing.

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Additional information
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