QMC-PCRx: a novel method for rapid mutation detection

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ABSTRACT

Aims We previously described the quick multiplex consensus PCR (QMC-PCR) as a method for rapid mutation screening in low-quality template. QMC-PCR has two-stages: a prediagnostic multiplex (PDM) reaction followed by a single specific diagnostic reaction with high-resolution melting (HRM) analysis. We aimed to develop QMC-PCRx in which second stage was multiplexed to allow testing of multiple targets.

Methods The PDM reaction was retained without change. For the second stage, *in silico* design was used to identify targets amenable to a multiplex specific diagnostic reaction and multiplex HRM (mHRM) analysis. Following optimisation, 17 colorectal cancers were tested for mutation in five hotspots. For QMC-PCR, each target was tested individually. For QMC-PCRx, the targets were tested in the following combinations (i) *KRAS* exon 3/*PIK3CA* exon 20/*PTEN* exon 3 in triplex and (ii) *PTEN* exon 7/*NRAS* exon 2 in duplex. The degree of agreement between the novel QMC-PCRx and the standard QMC-PCR was tested by the percentage concordance.

Results Optimisation of mHRM showed that peaks needed to be separated (without overlap) and the optimal number was three targets per test. Our experimental design produced distinct and widely separated peaks for the individual targets although one of the primers needed a GC-tail. A total of 85 individual targets were tested; this required 85 second-stage PCR/HRM tests by QMC-PCR versus 34 second-stage tests by QMC-PCRx. The percentage concordance between the singleplex and multiplex methodologies was 100%.

Conclusions A multiplexed analysis using HRM is possible without loss of diagnostic accuracy. The novel QMC-PCRx protocol can significantly reduce workload and costs of mutation screening.

INTRODUCTION

Tumours harbour multiple mutations that are acquired during tumourigenesis and tumour progression. The mutation profile (ie, the driver mutations found within a tumour) can be used for molecular subtyping and it may have prognostic and therapeutic importance clinically.[1–3] For example, in colorectal cancer (CRC), patients whose tumours have the *KRAS* and *BRAF* mutations have worse disease outcomes than patients lacking these mutations (ie, they are of prognostic significance) and are resistant to anti-EGFR therapy (ie, they are of predictive significance).[4 5] Mutation profiling of cancer can therefore be an invaluable clinical tool for defining diagnostic categories and for stratifying patients into prognostic and therapeutic groups.

Previously we described the quick multiplex consensus PCR (QMC-PCR) followed by high-resolution melting (HRM) analysis for rapid mutation screening from poor-quality templates such as formalin-fixed, paraffin-embedded (FFPE) tissue.[6 7] Formalin fixation with paraffin embedding is a universal method of preserving patients' tissue in pathology department archives but it does render it difficult to amplify by PCR.[8–10] The value of the QMC-PCR method therefore lies in its ability to deliver good-quality amplification of targets from FFPE DNA samples. The QMC-PCR is a nested PCR comprising two stages: the prediagnostic multiplex (PDM) and the single specific diagnostic (SSD) stages. In the PDM stage, approximately 10 targets are amplified in multiplex PCRs using outer primer pairs, while in the SSD stage specific targets are amplified in singleplex reactions using inner pairs and the diluted products of the PDM stage as template. The products of the SSD are subsequently analysed individually by HRM for mutation. The QMC-PCR protocol is cheap, fast and simple to operate and, since its description, has found applications in cancer research and plant genetics.[11 12]

Here we present an improvement of the QMC-PCR method that has been driven by a desire to reduce manpower and consumable costs. We have called this QMC-PCRx and the first-stage PDM is retained as previously described. Our development involves testing multiple targets in the second-stage reaction—converting SSD into the multiplex specific diagnostic (MSD) stage and evaluating multiple targets in the HRM analysis (ie, multiplex HRM, mHRM). The protocol and combination of multiplexed targets were designed *in silico* and the degree of agreement between QMC-PCR and QMC-PCRx was tested by crude percentage concordance on a series of cases with known mutations. Our protocol is different from other mHRM protocols inasmuch as it is specific for FFPE tissue-derived template. It involves modification of primers where necessary and it describes clear parameters for reliable mHRM mutation detection.

MATERIALS AND METHODS

Ethical approval for this study was granted by the Nottingham Health Sciences Biobank. Seventeen FFPE CRC cases from the pathology archives of the Queen's Medical Centre were included in this study.

DNA extraction

DNA was obtained from two main sources. Fresh DNA was used for the optimisation of the

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Table 1 List of PCR primers used for the optimisation of mHRM analysis

Gene	Primer sequence	Product size (bp)	Predicted Tm (°C)
TP53 E8b	F: GCCTGTCCTGGGAGAGAC R: CCTTTCTTGCGGAGATTCT	53	84.3
TP53 E4d	F: GGCAGCTACGGTTTCC R: CCCTCAGGGCAACTGA	80	87.8
SMAD4 E9a	F: GTATTGGTGTTCCATTGCTTACT R: TAAATGTCTCTCCTACCTGAACAT	56	76.9

mHRM, multiplex high-resolution melting; Tm, melting temperature.

methodology and was derived from the CRC cell lines Vaco5, SW948, HCT 116, SW620 and Vaco10MS. These were a kind gift from Professor Ian Tomlinson and had been previously validated by mutation profiling.[6]

In order to test the methodology, DNA was used from 17 cases of CRC. The FFPE tissue from these cases was processed using the QIAamp DNA FFPE Tissue Kit with some minor modifications to the protocol. Sections of 20 µm thickness were cut and placed in 1.5 mL Eppendorf tubes. Deparaffinisation was achieved via the addition of mineral oil to the tube with incubation at 80°C for 1 min. Tissue lysis and protein digestion were run with an overnight incubation at 56°C in a thermomixer (which was set at 350 rpm) to ensure adequate tissue digestion. The protein digestion was followed by DNA precipitation with 100% ethanol in buffer AL. The precipitated DNA was then bound to the MinElute column, following which the DNA, bound to the column, underwent a two-step washing process. Elution of the bound DNA was achieved via the addition of 40 µL ATE buffer to the column with a 5 min incubation step at room temperature. All other steps remained consistent with the Qiagen protocol.

Polymerase chain reaction

PCR for all reactions was performed on either ABI 7500 Fast PCR or the Strategene MxPro 3005 P PCR machine. Reaction conditions are described below and primer sequences are shown in tables 1–3.

Table 2 List of PCR primers used to demonstrate how to achieve adequate separation of Tms of multiplex targets

Gene	Primer sequence	Product size (bp)	Predicted Tm (°C)
KRAS E2 inner	F: ATATAAACTTGTGGTAGTTGGAG R: TATCGTCAAGGCACTCTTGC	62	84.1
KRAS E3 tailed	F: cccgggcgggccggcccCTTGGATATTCTCGACACAGCA R: cccgggcgggccggcccTCCCTCATTGCACTGTACTCCT	85	92.0
KRAS E4 inner	F: TGGAATTCCTTTTATTGAAACATC R: TTTCAGTGTTACTTACCTGTCTTGTCT	56	75.1
BRAF E11 inner	F: TGGGCAGATTACAGTGGGA R: GCCACTTTCCCTTGTAGACTG	68	79.6
BRAF E15 tailed	F: gggccggcccTTCATGAAGACCTCACAGTAAA R: gggccggcccGACCCACTCCATCGAGAT	90	85.9

The tailed primers for KRAS exon 3 and *BRAF* exon 15, as well as other primers used for the optimisation of the MSD reactions, were 'manually' designed using a combination of UCSC *in silico* PCR and MFEprimer web-based software.[13 14]
MSD, multiplex specific diagnostic; Tm, melting temperature.

HRM analysis

The PCR amplicons were subjected to HRM and analysis on the LightScanner-96 platform. The products were first transferred into wells in the LightScanner HRM plate, and a 20 µL mineral oil overlay was placed in each well. The PCR amplicons were then spun down in a Megafuge centrifuge for 5 min at 2500 rpm. Melting was performed using the following parameters: the temperature range of the instrument was set at 65°C–95°C and to 'Auto' exposure with a ramp rate of 0.1°C/s. Sample equilibration was performed at 62°C. The acquired melting data were analysed with the LightScanner Call-IT software V.2.0.0.1.331 using the Expert scanning module. The negative filter was used to exclude the negative controls and poorly amplified samples from further analyses. Normalisation

Table 3 Primer sequences used for the PDM, MSD and SSD reactions

Gene	Primer sequence	Product size (bp)	Predicted Tm (°C)
KRAS E2 outer	F: TGAATATAAACTTGTGGTAGTTGG R: GCTGTATCGTCAAGGCACTCT	174	NA
KRAS E2 inner	F: ATATAAACTTGTGGTAGTTGGAG R: TATCGTCAAGGCACTCTTGC	62	84.1
KRAS E3 outer	F: CCAGACTGTGTTTCTCCCTTC R: AAAGAAAGCCCTCCCCAGT	152	NA
KRAS E3* inner	F: TGTGTTTCTCCCTTCTCAGGA R: AAGAAAGCCCTCCCCAGT	145	86.3
KRAS E4 outer	F: AGACACAAAACAGGCTCAGGA R: TTGAGAGAAAAACTGATATATTAAATGAC	160	NA
BRAF E15 outer	F: ATCTACTGTTTTCCTTTACTTACTACAC R: CAGCATCTCAGGGCCAA	205	NA
PIK3CA E1 outer	F: CACGACCATCATCAGGTGAA R: GGAGGGGGTATTTTCTTGCT	168	NA
PIK3CA E9 outer	F: CTGTGAATCCAGAGGGGAAA R: GCACTTACCTGTGACTCCATAGAA	197	NA
PIK3CA E20 outer	F: TGAGCAAGAGGCTTTGGAGT R: CCTATGCAATCGGTCTTTGC	201	NA
PIK3CA E20 inner	F: GCAAGAGGCTTTGGAGTATTTC R: TTTTCAGTTCAATGCATGCTG	115	81.9
PTEN E3 outer	F: TCATTTTTGTTAATGGTGGCTTT R: ACTCTACCTCACTCTAACAAGCAGA	182	NA
PTEN E3 inner	F: GGCTTTTTGTTTGTTTGTTTTG R: CCTCACTCTAACAAGCAGATAACTTTC	158	78.4
PTEN E5A outer	F: GGTTATCTTTTTACCACAGTTGCAC R: GATTGTCATCTTCACTTAGCCATT	118	NA
PTEN E7 outer	F: GTTCCCTCAGCCGTTACCT R: CACCTGCAGATCTAATAGAAAACAA	191	NA
NRAS E2 outer	F: GGTTTCCAACAGGTTCTTGC R: TCCGACAAGTGAGAGACAGG	191	NA
NRAS E2 inner	F: TACAAACTGGTGGTGGTTGG R: CACTGGGCCTCACCTCTATG	115	86.6

*These primers were tailed and used for the MSD reactions.
The outer and inner primers were designed using *Primer3* as previously described.[6]
These primers have also been used in previous studies.[6] The *KRAS* exon 3 and *BRAF* exon 15 tailed primers were used for the MSD reactions.
MSD, multiplex specific diagnostic; NA, not applicable; PDM, prediagnostic multiplex; SSD, single specific diagnostic.

of the melting data was done as per manufacturer's instructions. The shifting level was set to 0.05, while sensitivity was set to normal at zero level. Both the 'Auto group' and 'Common vs Variant' functions were used to group the normalised and shifted melting curves according to their melting patterns. The results were viewed in the 'Shifted melt curves' and 'Difference curves' outputs.

Principles of the mHRM analysis and its impact on primer design and target combinations

HRM is used to detect variation in the DNA sequence of PCR amplicons and is dependent on (i) sequence dependent-variation in the physical properties of the DNA and (ii) formation of heteroduplexes when there is allelic variation in the starting template. Usually only one target is evaluated in each HRM analysis and the melting pattern of the target is plotted against the melting DNA pattern of a reference sample (of known sequence) generating a 'difference plot'. We reasoned that multiple targets could be tested in a single HRM analysis provided the melting temperature (Tm) of each of the individual targets was sufficiently separated that it would not interfere with each other during the melting analysis. This set the following constraints in the second MSD stage of QMC-PCRx: (i) the combination of the targets had to produce the PCR amplicons with a widely separated Tm and (ii) the primers must work efficiently but must not interact with each other.

Optimisation of the mHRM analysis for detecting multiplex targets

In order to ascertain whether mHRM was possible, we optimised the system using targets in *TP53* and *SMAD4*. We used high-quality DNA derived from five CRC cell lines—Vaco5 (containing *TP53* E282W), SW948 (containing *TP53* codon 117, G del), HCT116, SW620 and Vaco10MS. The last three cell lines are wild type (WT) at the above-mentioned *TP53* loci while all five cell lines are WT at *SMAD4* exon 9a. We tested three different targets: *TP53* exon 8b (encompassing the codon 282; predicted Tm of 84.3°C, *TP53* exon 4d (encompassing codon 117; predicted Tm of 87.8°C) and *SMAD4* exon 9a (encompassing codon 330; predicted Tm of 76.9°C). The targets were tested in duplex in different combinations and the cycling conditions were as follows: 1 cycle of 95°C for 5 min followed by 45 cycles of 95°C for 5 s, 60°C for 30 s and 72°C for 20 s. The cycling was followed by a 5 min final extension, and a melt curve stage comprising 30 s each at 95°C, 55°C and 95°C. The PCR primer sequences for this optimisation step are shown in table 1. For the HRM analysis, as there were multiple peaks, data normalisation was achieved by placing the cursors astride individual peaks or raw melt curve inflections (see Results). Other melting and analysis parameters were the same as for the single product analysis (as described above).

GC-rich tailed primers for achieving adequate separation of target melt curves along the temperature line

Our initial optimisation experiments indicated that, for a reliable analysis by mHRM, the melting peaks of the individual PCR amplicons need to be separated without overlap (see Results). This is not always possible using the natural Tm of the PCR amplicons and thus we sought to modify the primers to enhance the differences. Bioinformatics analyses with UCSC *in silico* PCR (https://genome.ucsc.edu/cgi-bin/hgPcr), MFEprimer (http://www.biocompute.bmi.ac.cn/CZlab/MFEprimer-2.0/) and UMELT Batch 2.0 (https://www.dna.utah.edu/umelt/umb.php) were used in combination to demonstrate *in silico* that adding a GC-tail to some primers could be used to alter the Tm and thereby enhance separation of multiplex amplicons in order to ensure reliable mHRM.[13–15] This was confirmed experimentally and the primer sequences are shown in table 2.

Comparison of QMC-PCR and QMC-PCRx

PDM reaction

In order to compare the standard QMC-PCR with the new QMC-PCRx protocol, a total of 17 cases of CRC were tested using both protocols in parallel. Both of the protocols have a common PDM stage. Eleven targets comprising *PTEN* exons 3, 5 and 7, *KRAS* exons 2, 3 and 4, *NRAS* exon 2, *BRAF* exon 15 and *PIK3CA* exons 1, 9 and 20 were amplified with outer primer pairs in 20 μL multiplex reactions that consisted of 10 μL of Diamond Hotshot master mix, 1 μL of Eva Green dye, 400 nM of each outer primer and 20 ng of template. The cycling parameters included 1 cycle of 95°C for 5 min followed by 30 cycles of 95°C for 3 s and 55°C for 10 s. The PCR amplicons were diluted 1:100 in nuclease-free water and then used for the SSD and the MSD reactions.

QMC-PCRx: the MSD reactions and mHRM

Using the online UMELT software we predicted the targets with well-separated peak Tms, which would therefore be suitable for the mHRM analysis. Five targets were combined as follows for MSD/mHRM: *PTEN* exon 3, *PIK3CA* exon 20 and *KRAS* exon 3 were combined in triplex reactions while *PTEN* exon 7 and *NRAS* exon 2 were chosen for duplex reactions. Two microlitres of the diluted PDM reaction served as templates for the MSD reactions. One of the primer pairs, *KRAS* exon 3, was tailed in order to enhance the Tm separation.

PCR was undertaken in a final volume of 15 μL, and the primer concentration was 250 nM for all the exons except *NRAS* exon 2 for which a concentration of 75 nM was used. The cycling parameters for the MSD reactions were as follows: 1 cycle of 95°C for 5 min, followed by 45 cycles of 95°C for 10 s, 60°C (for the triplex) and 55°C (for the duplex) for 30 s and 72°C for 20 s. This cycling was followed by a final extension of 72°C for 5 min.

SSD reactions

Each of the five targets was tested individually using SSD/HRM. The template, the final volume and the primers (apart from the tailed *KRAS* exon 3 primers) were the same as MSD. All primers were used at a concentration of 250 nM and the cycling parameters were as follows: 1 cycle of 95°C for 5 min followed by 32 cycles of 95°C for 3 s and 55°C (62°C for *KRAS* exon 2) for 10 s. The primer sequences used for the PDM, SSD and MSD reactions are shown in table 3.

All PCRs were run in duplicates for each sample. Some of the MSD and SSD experiments were repeated two or three times to ensure reproducibility.

Statistical analyses

The HRM results of the SSD and MSD reactions were tabulated as either WT or mutant (M), and the degree of agreement between the two assays was tested using a simple calculation of the crude percentage concordance.

RESULTS

Significant melt curve overlap produces artefacts on the mHRM analysis

While the melt curves of *TP53* exons 4d and 8b amplicons overlapped with each other close to their peaks, both were each

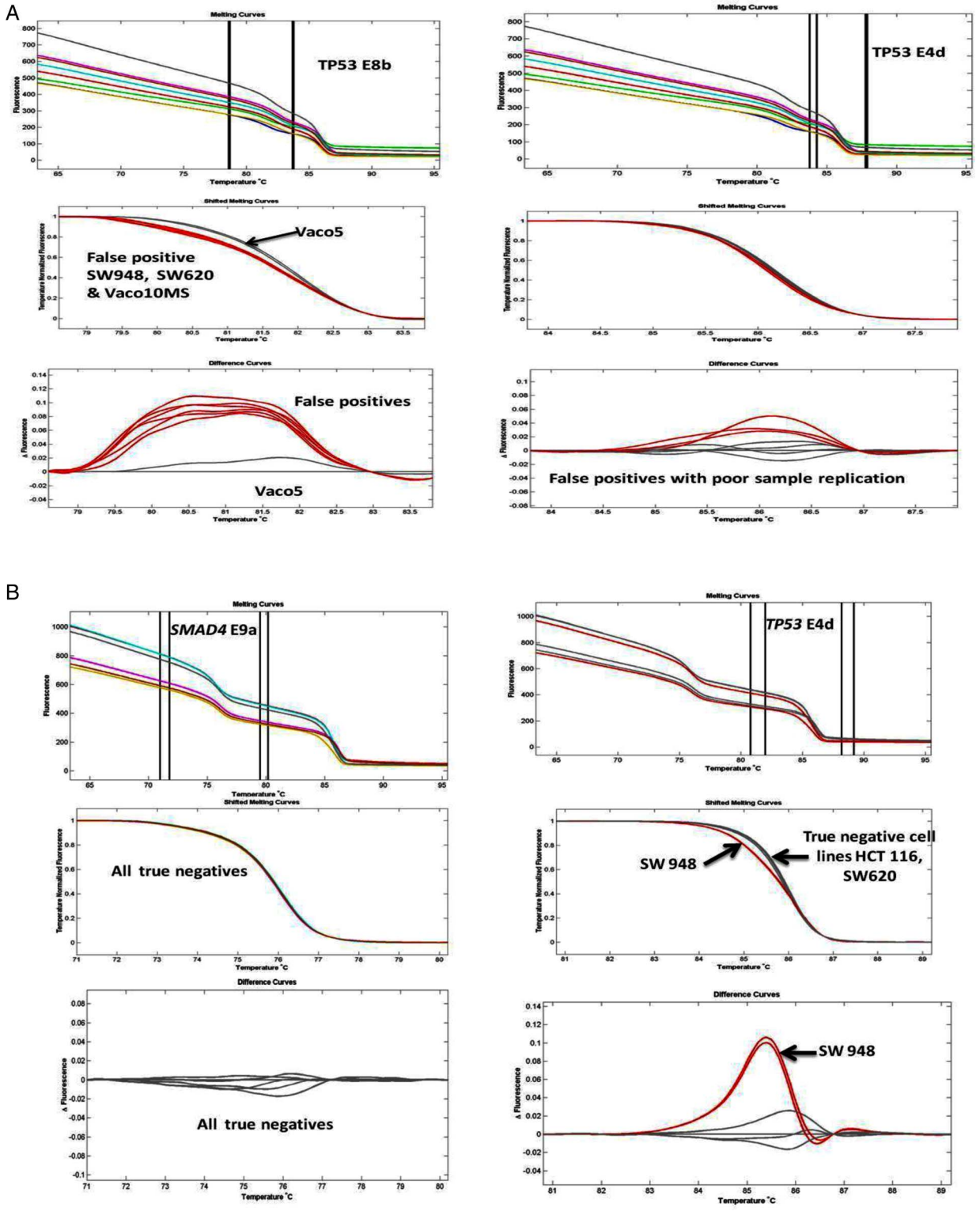

Figure 1 Effects of overlapping and well-separated melt curves on high-resolution melting analysis. The *TP53* E4d and *TP53* E8b melt curves overlap significantly (A and B) resulting in difficulty in cursor placement and analysis (B). The results of melt curve overlap include poor reproducibility of the analysis, false positivity and negativity (B). On the other hand, the *SMAD4* E9a and *TP53* E4d curves are well spaced apart (A and C) and cursor placement was easy, analysis was reproducible and results were reliable (C).

completely separate from the melt curve of the *SMAD4* exon 9a amplicon (see online supplementary figure S1). Normalisation of each peak was performed by placing the cursors astride individual raw melt curve inflections. With overlapping peaks, there was difficulty with attempts at normalisation of individual peaks or melt curves because of the short distance between the inflection of one target amplicon and that of the next amplicon (figure 1). The resulting shifted melt curves and difference

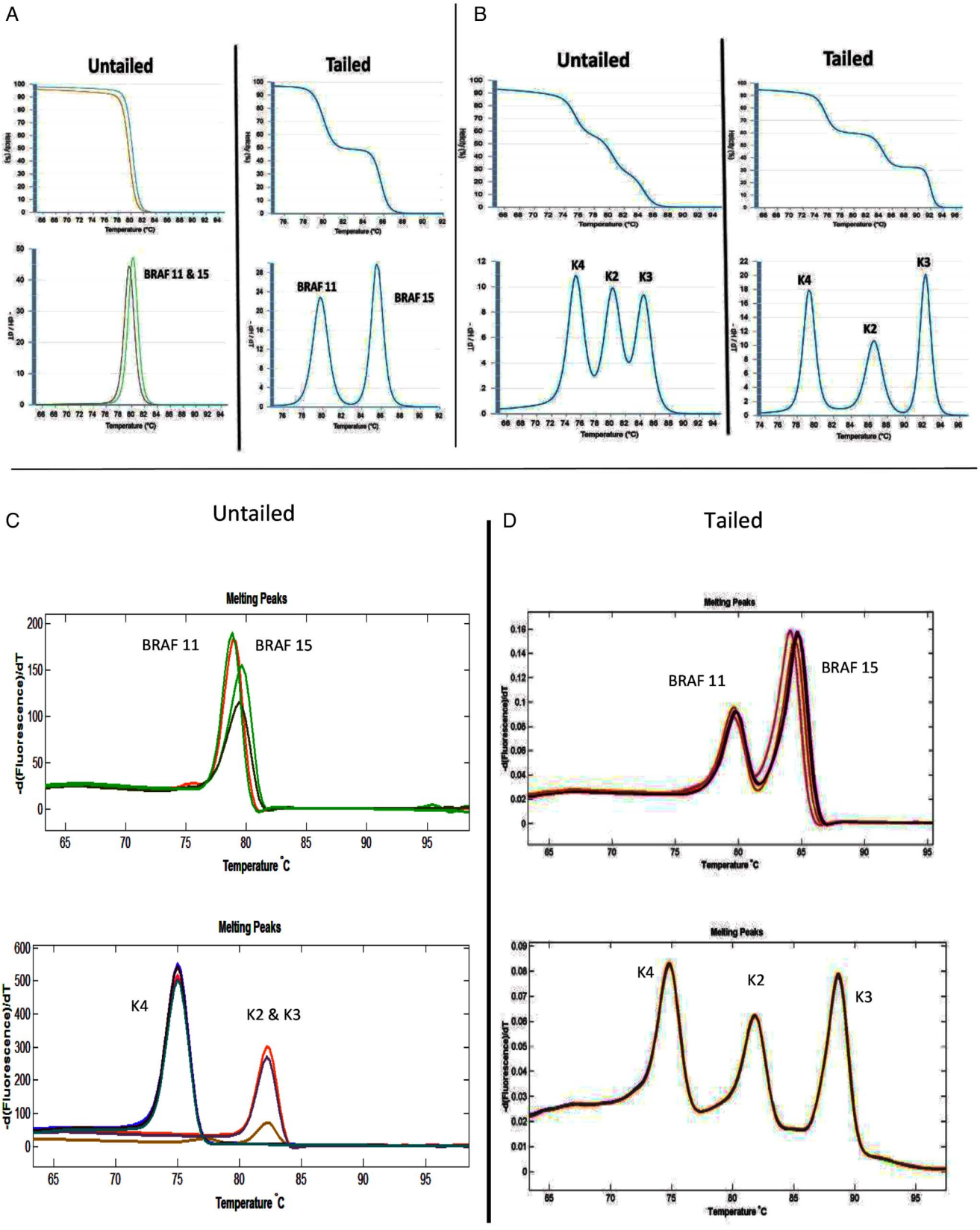

Figure 2 (A and B) *In silico* (on UMELT) demonstration of the capacity of GC-rich tailed primers to achieve adequate separation of melting Tms for the components of a multiplex reaction. K4, K2 and K3 are *KRAS* exon 4, *KRAS* exon 2 and *KRAS* exon 3, respectively. In this analysis, *BRAF* exon 15 and *KRAS* exon 3 primer pairs were tagged with GC-rich tails. (C and D) Experimental demonstration of the capacity of GC-rich tailed primers to achieve adequate separation of melting Tms for the components of a multiplex reaction. K4, K2 and K3 are *KRAS* exon 4, *KRAS* exon 2 and *KRAS* exon 3, respectively. The GC-rich tailed primer pairs were those of *BRAF* exon 15 and *KRAS* exon 3.

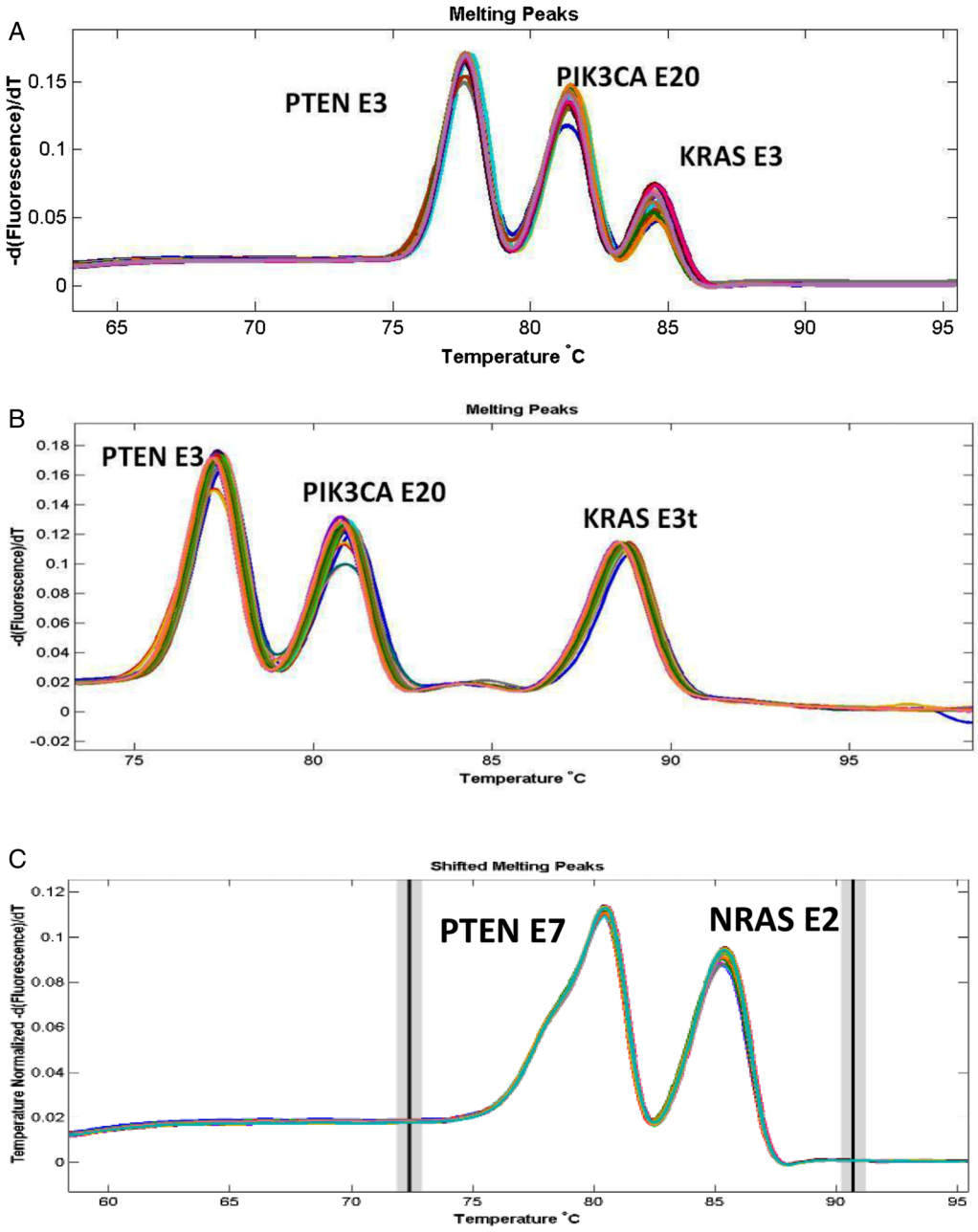

Figure 3 Multiplex specific diagnosis. (A and B) *KRAS* exon 3 tailed primers were used to achieve good separation of *PIK3CA* exon 20 and *KRAS* exon 3 amplicons. (C) The *NRAS* exon 2 and *PTEN* exon 7 amplicons showed good separation without primer tailing.

curves from multiplex targets with overlapping melt curves showed spurious aberrant melting results suggesting mutation in samples with known wild-type genotypes (figure 1). On the other hand, when the melt curves were sufficiently separate and there was minimal or no overlap between the curves, the Call-IT displayed the correct melting patterns for the samples (figure 1). The MSD reactions were therefore performed with primers that amplify amplicons with adequately separated Tms.

Use of GC-tagged primers enhances the separation of targets for robust mHRM

Our optimisation data showed that overlapping melting patterns resulted in artefacts making interpretation difficult and unreliable. We reasoned that adding non-specific GC-rich tails could alter the Tm of the PCR amplicons and thereby improve the mHRM analysis. We tested this *in silico* and then verified it experimentally with a triplex for *KRAS* exons 2, 3 and 4 and a duplex of *BRAF* exons 11 and 15. The primer pairs for *KRAS* exon 3 and *BRAF* exon 15 were tagged with non-specific GC-rich tails and used to perform *in silico* PCR using MFEprimer. The PCR amplicons of each multiplex group were pasted on UMELT Batch 2.0 mode and HRM analysis was performed *in silico*. The results of *in silico* analyses showed that the use of GC-rich tailed primers for PCR of one or more targets of the multiplex enhanced the separation of the melting domains for the multiplex reaction through the increases in Tm (figure 2A). The *in silico* predictions were tested and verified experimentally (figure 2B).

Assay agreement: QMC-PCR versus QMC-PCRx

Our data showed that, with appropriate amplicon selection and primer modification, multiple targets could be tested in the HRM

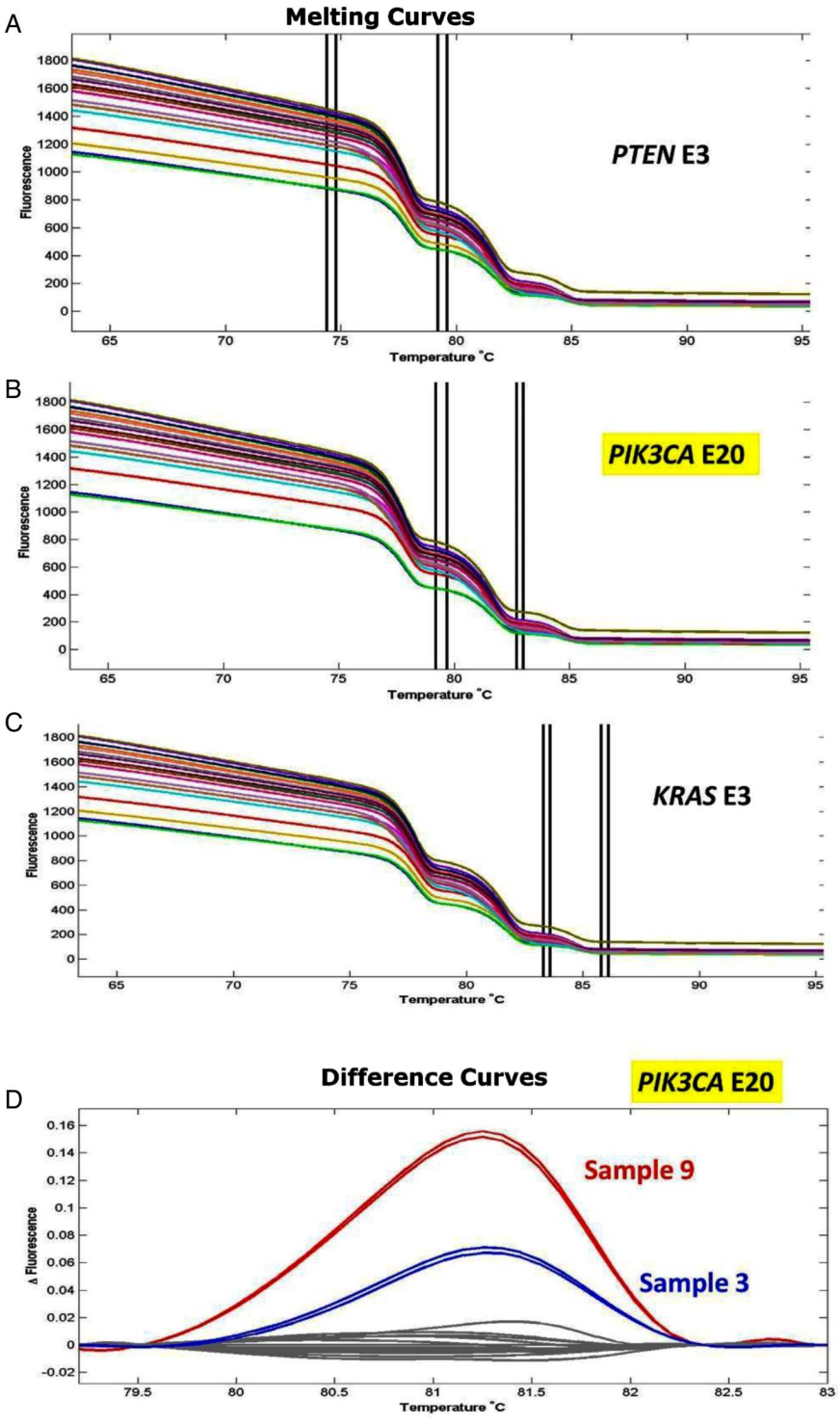

Figure 4 (A–C) Melt curve data normalisation for each amplicon. (D) Aberrant melting pattern of samples 3 and 9 at *PIK3CA* exon 20 was found with both the multiplex specific diagnostic and the single specific diagnostic reactions.

analysis. Five separate targets were tested in each of the 17 cases of CRC by QMC-PCR and QMC-PCRx. For QMC-PCRx, *PTEN* exon 3, *PIK3CA* exon and 20/*KRAS* exon 3 were combined in triplex reactions and *PTEN* exon 7 and *NRAS* exon 2 were combined in a duplex reaction. We designed nested primers that amplify short products (<150 bp long). The targets were grouped into multiplexes based on the Tm predictions, which were also confirmed experimentally. While for the duplex group the melt curve separation was adequate, there was significant Tm overlap between the *PIK3CA* exon 20 and *KRAS* exon 3 targets in the triplex group. Thus the primers for the *KRAS* exon 3 targets were redesigned to have GC-rich tails at the 5′ end and to amplify a product with a higher Tm and thereby separate melting of the *PIK3CA* exon 20 and *KRAS* exon 3 targets without significantly altering the common optimum annealing temperature of the multiplex PCR (figure 3). For QMC-PCRx, the target amplicons showed distinct melting peaks (figures 3–5). The HRM normalisation of the peaks or curve inflections was therefore successfully accomplished using the approach described in the Materials and methods section (figures 4 and 5). A total of 85 individual targets

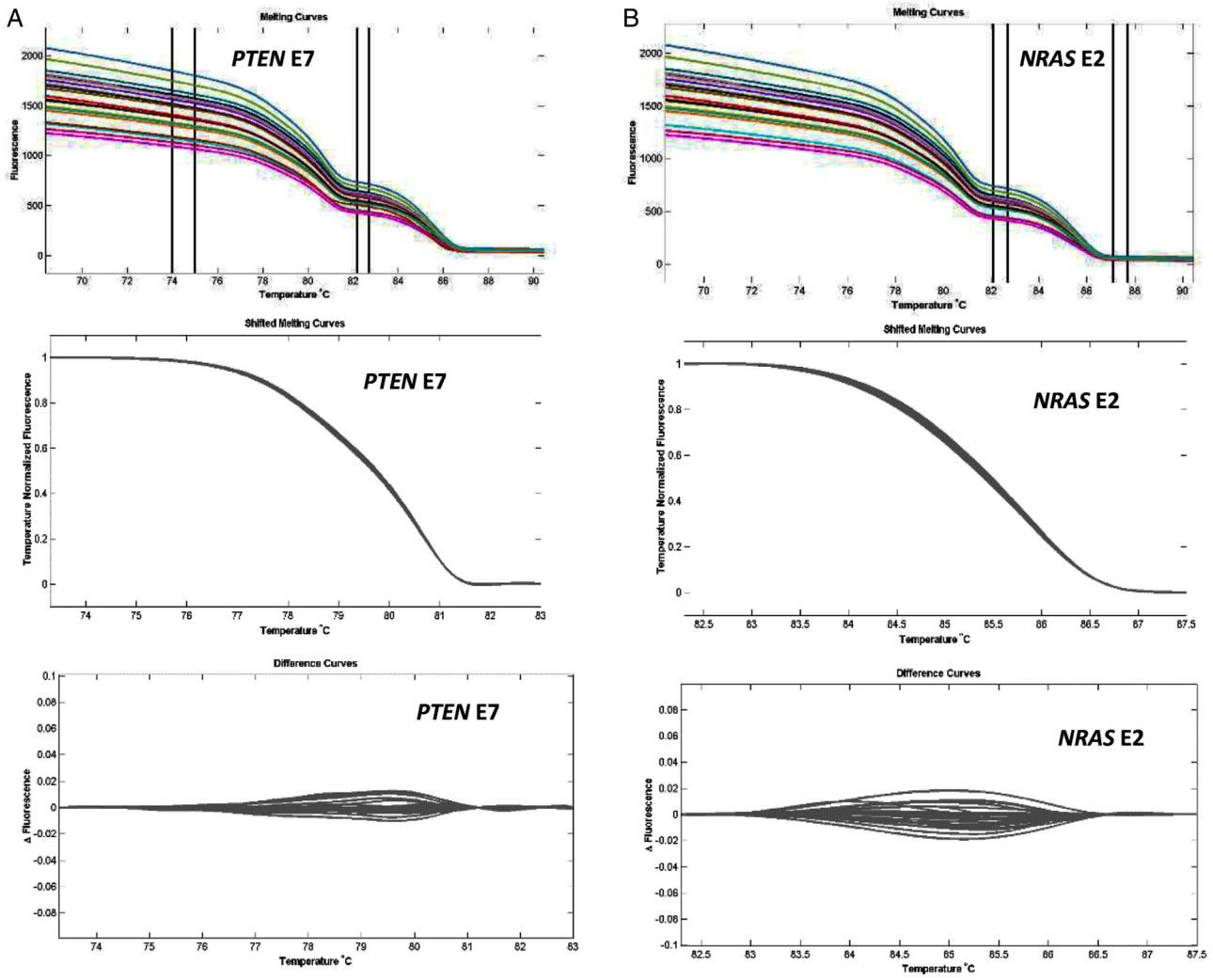

Figure 5 Multiplex specific diagnosis of *PTEN* exon 7 (A) and *NRAS* exon 2 (B) showing no mutations in either as shown by the shifted melting and difference curves.

were tested and there was absolute agreement between SSD and MSD on the HRM analysis, giving an overall crude percentage concordance of 100% (table 4). Furthermore, we did not find any differences between the two methodologies caused by differences in mutant allele frequency.

DISCUSSION

Our research group has previously shown that QMC-PCR is a robust and rapid way of screening for mutations.[7] We sought to improve this by using physical properties of DNA to allow multiplexing of the HRM analysis and thereby reduce the labour and consumable costs of the technique. We have shown that, with appropriate target choice and primer modification, this is indeed possible.

We compared our new protocol, which we call QMC-PCRx, with our old protocol on five targets tested in 17 cases of CRC (all of which were FFPE tumours). A total of 85 individual targets were tested and, using the standard QMC-PCR protocol, this required 85 second-stage SSD PCR and HRM tests. In contrast, with the new protocol, only 34 second-stage MSD PCR and HRM tests were required. There was 100% concordance between the two protocols showing that QMC-PCRx performs as well as the old protocol. This was just a pilot project with potentially a huge difference in the costs associated with the testing both for consumables and for manpower (see online supplementary data)—with further optimisation further cost reductions could probably be achieved.

Although QMC-PCRx does significantly increase the efficiency of the mutation analysis, it does have some caveats. First, as with all multiplex assays, it is a more complicated assay to set up than a singleplex assay. The targets chosen for each MSD reaction have to be chosen appropriately and, if necessary, primers may need modification. In this regard, the use of online bioinformatics tools aided the design of our multiplex assays. These tools are easy to use and accurate thereby significantly reducing the on-the-bench optimisation times. Specifically, the UMELT HRM prediction software enabled the prediction of Tms for the singleplex and multiplex melt curve Tms and guided our experimentation.[15] Additionally, the MFEprimer software enabled the design of modified (GC-tailed) primers since it accommodates such modification at the 5′ end as it performs *in silico* PCR.[14] The data in figure 2 show that there was near-identical performance of the experiments with the *in silico* predictions. Second, the mHRM analysis cannot be automated

Table 4 Comparison of MSD and SSD

Targets / Samples	*PTEN* E3		*PIK3CA* E20		*KRAS* E3		*NRAS* E2		*PTEN* E7	
	SSD	MSD	SSD	MSD	SSD	MSD	SSD	MSD	SSD	MSD
1	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
2	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
3	WT	WT	**M**	**M**	WT	WT	WT	WT	WT	WT
4	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
5	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
6	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
7	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
8	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
9	**M**	**M**	**M**	**M**	WT	WT	WT	WT	WT	WT
10	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
11	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
12	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
13	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
14	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
15	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
16	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
17	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT

The samples and targets in bold denote the presence of mutation "M".
M, mutant; MSD, multiplex specific diagnostic; SSD, single specific diagnostic; WT, wild type.

as it currently can be done with a single-target analysis. In our experience, an automated software analysis always needs checking manually, and so there would be little time lost during the analysis. Even if the automated analysis is perfectly reliable for singleplex reactions, the savings engendered by the reduction in the number of PCR tests would hugely outweigh the costs of manual analysis. Only missense mutations were tested in this analysis although different types of mutation can occur (such as insertions and deletions). HRM can however detect different types of mutations (for insertions/deletions, data interpretation is often easier) and we would not expect any loss in performance when testing for these types of mutations.

As HRM analysis uses a single dye and depends on differences in melting temperature profiles of amplicons, mHRM multiplexing is only achievable by varying the Tm of the target amplicons.[16] We have shown that, when there is a significant overlap of the melting curves of the multiplex amplicons, the HRM analysis is unreliable (figure 1). Well-spaced curves assure a reliable analysis but at the cost of a reduction in the number of potential targets. We believe that three targets can be reliably tested and it may be possible to push this up to four.

Multiplexing of targets for HRM has previously been tested by different research groups using diverse strategies to achieve adequate spacing of target amplicons. While Seipp *et al*[17] used GC-rich and AT-rich tailed primers as well as primers incorporating locked nucleic acids to achieve ample gaps between the melt curves of the target amplicons, Pereyra *et al*[18] designed conventional, non-modified primer pairs to amplify targets of 50–200 bp long, simply using product sizes to achieve adequate separation of melt curve Tms. These have been reasonably successful but they are single-stage reactions which increases the risk of inappropriate primer interaction. QMC-PCRx is a nested protocol which, due to the PDM stage, markedly reduces the chances of non-specific priming in the MSD reaction.

In conclusion, we have extended our previous work to produce the QMC-PCRx protocol. This depends on choosing the right combination of targets and primer modifications to allow mHRM analysis. It performs as reliably as the QMC-PCR method but will hugely reduce cost, workload and the turnaround time of research.

Take home messages

- A multiplexed analysis using high resolution melting is possible without loss of diagnostic accuracy.
- The novel QMC-PCRx protocol can significantly reduce workload of mutation screening.
- The novel protocol is more cost-effective than the old QMC-PCR for mutation screening.

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