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Base-pair neutral homozygotes can be discriminated by calibrated high-resolution melting of small amplicons

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ABSTRACT

Genotyping by high-resolution melting analysis of small amplicons is homogeneous and simple. However, this approach can be limited by physical and chemical components of the system that contribute to intersample melting variation. It is challenging for this method to distinguish homozygous G::C from C::G or A::T from T::A base-pair neutral variants, which comprise ~16% of all human single nucleotide polymorphisms (SNPs). We used internal oligonucleotide calibrators and custom analysis software to improve small amplicon (42–86 bp) genotyping on the LightScanner®. Three G/C (*PAH* c.1155C>G, *CHK2* c.1-3850G>C and candidate gene *BX647987* c.261+22,290C>G) and three T/A (*CPS1* c.3405-29A>T, *OTC* c.299-8T>A and *MSH2* c.1511-9A>T) human single nucleotide variants were analyzed. Calibration improved homozygote genotyping accuracy from 91.7 to 99.7% across 1105 amplicons from 141 samples for five of the six targets. The average T_m standard deviations of these targets decreased from 0.067°C before calibration to 0.022°C after calibration. We were unable to generate a small amplicon that could discriminate the *BX647987* c.261+22,290C>G (rs1869458) SNP, despite reducing standard deviations from 0.086°C to 0.032°C. Two of the sites contained symmetric nearest neighbors adjacent to the SNPs. Unexpectedly, we were able to distinguish these homozygotes by T_m even though current nearest neighbor models predict that the two homozygous alleles would be identical.

INTRODUCTION

Genotyping by melting traditionally relies on the melting temperature (T_m) of a labeled probe hybridized to an amplification product (1–3). The primary advantage of probe-based genotyping is that the T_m between the probe and the target sequences in the two alleles usually varies by 2–8°C and is easily detected by standard methods. This large T_m separation occurs with probes of 15–35 bases that are perfectly matched to one allele.

High-resolution melting analysis of amplicons is an attractive genotyping method because it eliminates the need for oligonucleotide probes (4,5). Only two standard PCR primers are used and no sample processing is required after amplification is begun. While heterozygotes are easily detected, discriminating between homozygous alleles is harder because the homozygotes display similar melting curves and T_m. For example, the homozygote of the common cystic fibrosis mutation F508del could not be distinguished from the wild-type allele using a 277-bp amplicon (6). Melting data are additionally confounded in microtiter plate-based systems by well-to-well variations due to hardware (instrument and plates) and chemistry factors (7–9).

High-resolution melting of small amplicons (~40–90 bp) improves homozygote detection sensitivity because T_m differences are greater compared to larger amplicons (4). Small amplicon melting clearly resolves homozygous alleles in at least 84% of human single nucleotide variants where one allele is an A::T pair and the other is a G::C pair, resulting in T_m differences of 0.8–1.4°C (10). It is more challenging to use small amplicon melting to genotype the ~16% of single base variants that are base-pair neutral when the GC content remains the same. In these G/C or A/T variants, T_m values are predicted to change by

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0.4°C or less (10). Furthermore, current nearest neighbor analysis (11) predicts no T_m difference in 4% of human single base variants with symmetric nucleotides flanking a base-pair neutral change, for example,

5′-... TGA ...-3′ changing to 5′-... TCA ...-3′
3′-... ACT ...-5′ 3′-... AGT ...-5′.

The homozygous alleles of *HFE* 187C>G contain these specific nearest neighbor symmetric sequences and were not distinguishable using small amplicon melting without mixing in a known homozygous control sample (12). In such a mixture, if only homoduplexes are observed, the genotype of the test sample is identical to that of the known standard. In contrast, if a heterozygous melting curve is obtained, the genotype of the test sample is different from that of the known sample.

Internal oligonucleotide calibrators can improve genotyping by melting. Molecular beacons have been used to normalize T_m across a microfluidic platform (13) and to minimize SYBR® Green I T_m variation across a 96-well plate (14). The use of two double-stranded oligonucleotide internal calibrators flanking a target amplicon improved genotyping of nonbase-pair neutral variants (i.e. G or C to A or T variants) in amplicons on both plate and capillary platforms (15,16). In each case, custom software normalized the T_m of the internal oligonucleotide calibrators across reactions for more accurate classification of genotypes.

Herein, we report the genotyping of six base-pair neutral human variants using small amplicon melting with two internal oligonucleotide calibrators on the 96-well LightScanner. Three of the variants consist of A::T to T::A exchanges located in the carbamyl phosphate synthetase gene (*CPS1* c.3405-29A>T), the ornithine transcarbamylase gene (*OTC* c.299-8T>A) and the mutator homolog gene (*MSH2* c.1511-9A>T). The variant sites in *CPS1* and *OTC* are nearest neighbor symmetric. The other three variants consist of G::C to C::G variants located in the phenylalanine hydroxylase gene (*PAH* c.1155C>G), the checkpoint 2 gene (*CHK2* c.1-3850G>C) and the candidate *BX647987* gene (c.261+22,290C>G).

MATERIALS AND METHODS

Human genomic DNAs

One hundred and forty-one human genomic DNA samples were obtained from healthy donors and de-identified according to an institutional review board approved protocol #7275. The DNA was isolated from whole blood using the PUREGENE® Genomic DNA Purification Kit from Gentra systems (Minneapolis, Minnesota, MN USA). After extraction, samples were adjusted to a final concentration of 10–15 ng/ul.

The genotypes of all DNA samples were confirmed using probe-based assays (3). In addition, representative samples of each genotype were sequenced from longer *OTC* and *CPS1* PCR products to confirm genotype.

Calibrators

Amplicon melting data were aligned (i.e. calibrated) relative to internal oligonucleotide calibrators of low (T_m ~62°C) and high (T_m ~92°C) T_m, henceforth referred to as low and high calibrators. Calibrators included in each reaction vessel provided PCR-independent melting signatures. The sequence of the low calibrator was 5′-TTAAATTATAAAATATTTATAATATTAATTATATATATATAAATATAATA-C3-3′. The sequence of the high calibrator was 5′-GCGCGGCCGGCACTGACCCGAGACTCTGAGCGGCTGCTGGAGGTGCGGAAGCGGAGGGGCGGG-C3-3′. These oligonucleotides, and their reverse complements, were included at 0.05 μM in each amplification reaction.

Importantly, all calibrators were blocked on their 3′-hydroxyl termini with a three-carbon (C3) alkyl group during synthesis to prevent extension by *Taq* polymerase. Fluorescent probes blocked with such alkyl moieties resist nucleotide extension by *Taq* polymerase after multiple freeze–thaw cycles and/or long-term storage better than phosphorylated probes (17).

Calibrators were obtained from IDT (Integrated DNA Technologies, Coralville, IA, USA) using standard phosphoramidite synthesis. After standard synthesis, PAGE purification was used to obtain higher oligonucleotide purity. Cartridge purification and reverse HPLC purification were also assessed with less optimal results.

Primer synthesis and primer design

Amplification primers were obtained from the University of Utah (Salt Lake City, UT, USA) Core Oligonucleotide/Peptide Synthesis Facility using standard phosphoramidite synthesis.

Primer sequences were analyzed (http://genome.ucsc.edu/cgi-bin/hgPcr) to minimize the likelihood that undesired products would co-amplify and interfere with the target sequence melting curves. Table 1 shows the variants, PCR primer sequences, concentrations and amplicon lengths. Some of the small amplicon PCR primers were designed to tightly flank the variants of interest leaving only 3–7 bases, including the polymorphism, between the primers. This is optimal for homozygote detection. However, the forward primer of the 68-bp *OTC* amplicon does not tightly flank the variant. This is due to the 7-bp long polythymidine tract followed by a cytidine and another 10-bp polythymidine tract immediately preceding the c.3405-29A/T variant. Other primers were constructed with up to 43 bp between each primer, for a total amplicon length reaching 98 bp.

PCR conditions and high-resolution melting

PCR was performed in 10 μl using 1X LightScanner® High Sensitivity Master Mix (Idaho Technology), containing LCGreen® Plus, and 0.05 μM of each internal oligonucleotide calibrator. In addition, 0.10 μM or 0.15 μM of primers (depending on target) and 10–15 ng of human genomic DNA were included in each reaction. PCRs were performed on iCyclers (Bio-Rad, Hercules, CA, USA) with a final $MgCl_2$ concentration of 2 mM. PCR conditions

Table 1. Genetic variants assessed and amplification primers utilized

Gene	Variant	Primers[a]	Primer length	Base pairs genotyped by melting[b]	Concentration (μM)	Total amplicon length (bp)
CPS1	c.3405-29A/T	AGTCAAGTCTAGTATTAGCATAAACCT	27	3	0.10	51
Acc # NM_001875	rs3213784	AAGGAAGGGGAAAAAAAGCAG	21		0.10	
OTC	c.299–8T/A	TCCACTTTAGTTGTTTTTTCAAAATGAT	28	20	0.10	68
Acc # NM_000531	rs not assigned	CCCAGAAGTGCAAAGCCTAC	20		0.10	
MSH2	c.1511-9A/T	TTTATGGAATACTTTTTCTTTTCTTC	26	4	0.15	49
Acc # NM_000251	rs12998837	AGGGTCCAAGCCTTGATAA	19		0.15	
PAH	c.1155C/G	AAATTACACTGTCACGGAGTTCCA	24	7	0.10	59
Acc # NM_000277	rs772897	CATCATTAAAACTCTCTGCCACGTAATA	28		0.10	
CHK2	c.1-3850G/C	CACCCATGCTTGCTATCTG	19	1	0.25	42
Acc # NM_001005735	rs9608698	GGCTTTCCAATAGCAATAGCTC	22		0.25	
CHK2	c.1-3850G/C	CCACCCATGCTTGCTATCT	19	11	0.25	50
Acc # NM_001005735	rs9608698	TCTGCATGGCTTTCCAATAG	20		0.25	
CHK2	c.1-3850G/C	AGTGAAGTGACGCATGTAATACTC	24	42	0.25	86
Acc # NM_001005735	rs9608698	ACTTCTCTGCATGGCTTTCC	20		0.25	
BX647987		AAGCCATAAGGTTAAACT	18	23	0.25	58
(uc003hum.1)	rs1869458	GGAAACCTACAGGATCA	17		0.25	
BX647987		AAACCTAAGGATGTTTTATGACATAATTTCTTG	33	43	0.25	98
(uc003hum.1)	rs1869458	TACAGCTGGAAACCTACAGGAT	22		0.25	

[a]Oligonucleotides oriented 5′ to 3′.
[b]This is the number of base pairs between the 3′ ends of the primers. In practice, at least the last and penultimate 3′ bases on both primers must match the target sequence for efficient amplification, but these potential allele-specific amplification affects are not considered.

Table 2. Thermal cycling and premelting protocols

Gene/Amplicon (bp)	Initial denature		Denature		Anneal		Cycles	Premelting protocol			
	Time (min:s)	Temp (°C)	Time (min:s)	Temp (°C)	Time (min:s)	Temp (°C)		Time (min:s)	Temp (°C)	Time (min:s)	Temp (°C)
CPS1 51 bp	2:00	95	0:30	94	0:30	66	45	0:30	94	0:30	28
OTC 68 bp	”	”	”	”	”	65	50	”	”	”	”
MSH2 49 bp	”	”	”	”	”	64	44	”	”	”	”
PAH 59 bp	”	”	”	”	”	67	45	”	”	”	”
CHK2 42 bp	”	”	”	”	”	64	45	”	”	”	”
CHK2 50 bp	”	”	”	”	”	66	45	”	”	”	”
CHK2 86 bp	”	”	”	”	”	66	45	”	”	”	”
BX647987 58 bp	”	”	”	”	”	63	45	”	”	”	”

were as follows: an initial denaturation of 95°C for 2 min, followed by 44–50 cycles (depending on the assay) of 94°C for 30 s and annealing at 63–67°C (depending on the assay) for 30 s. The exact thermocycling conditions are shown in Table 2. After completing amplification, a final denaturation and reannealing protocol was performed by raising the temperature to 94°C for 30 s followed by a 28°C hold for 30 s.

Following PCR, high-resolution melting was performed in a 96-well plate LightScanner (Idaho Technology) collecting data from 55–97°C at a ramp rate of 0.10°C per second. Calibration algorithm and genotype prediction.

Melting profiles were calibrated by first using a smoothing spline to approximate fluorescence data. Next, the T_m values were computed for each internal oligonucleotide calibrator present using the interpolated apex of the derivative spline data. Shift and linear scale factors were applied to align each calibrator. This shifting process aligns the amplicon melting profiles and minimizes variation. Data were re-sampled with a cubic spline fit and analyzed with commercial LightScanner software. The temperature regions surrounding the amplicon T_m were analyzed before and after prior alignment of the calibrator (+/– calibration) and displayed as derivative peaks. The probability that the observed T_m separation of alternative homozygotes was obtained by chance was assessed using the nonparametric Mann–Whitney U-test in Matlab® (The Mathworks, Inc., Natick, MA, USA).

Homozygous genotypes were automatically predicted by software before and after calibration based on the T_m values and standard deviations within each plate. We assumed that T_m values were normally distributed, that the estimated means were representative of the true population means, and that the variation was equal and representative for both populations. Homozygotes were classified by determining the distance from each sample to

the mean T_m value of each homozygote population within the plate. Since the genotypes of all samples were predetermined by probe-based methods and/or sequencing, we could determine whether each sample's T_m was closer to the correct or incorrect homozygous group. If the sample's T_m was closer to the mean T_m of the correct group, a successful classification occurred.

RESULTS

We designed and developed small-amplicon melting assays to genotype three G/C and three T/A variants in at least 47 unique human DNA samples and compared the results to probe-based assays and Sanger sequencing. The melting curves for all six base-pair neutral variants are shown in Figures 1–3. Figure 1 displays a complete derivative melting profile of the 42-bp *CPS1* c.3405-29A>T small amplicon before (A) and after (B) calibration. Melting signatures from the low and high oligonucleotide calibrators are observed at ~62°C and 92°C flanking the small amplicon melting peaks. The insets show magnified views of the homozygote peaks that are only well separated after calibration. Figure 2 shows similar small amplicon melting data for the *OTC*, *MSH2*, *PAH* and *BX647987* variants, while *CHK2* variant genotyping is shown in Figure 3. With the exception of the *BX647987* variant, calibration resolves the genotypes of the homozygous alleles. Five of the small amplicons, including *BX647987*, had two melting peaks for the heterozygous genotypes. Only the heterozygous 68-bp amplicon of *OTC* exhibited a single peak.

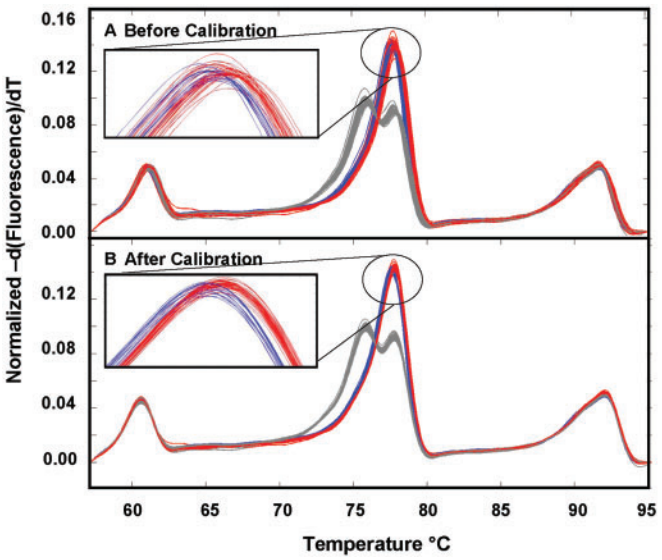

Figure 1. Derivative melting curves showing internal oligonucleotide calibrators and amplicon melting peaks for a target in *CPS1*. Ninety-four profiles, each generated from independent PCR reactions of 47 human genomic DNAs performed in duplicate, are shown before (**A**) and after (**B**) calibration. The blue and red peaks in the center represent the A/A and T/T genotypes, respectively, of the *CPS1* c.3405A/T polymorphism. The gray dual-peaks are from the A/T heterozygotes. Peaks from the low and high calibrators are on the left and right, respectively. Magnified apexes of the homozygote peaks are shown in the insets.

A summary of the T_m results for all variants is shown in Table 3. Predicted T_m values are listed for all homozygous alleles based upon nearest neighbor calculations (11,18). T_m values for heterozygotes are not included because they are, in general, easily genotyped without calibration. For our *MSH2* (49 bp), *PAH* (59 bp), *CHK2* (42 bp) and *BX647987* (58 bp) small amplicons, nearest neighbor analysis predicted T_m differences of 0.19°C, 0.18°C, 0.31°C and 0.04°C, respectively, between amplicons containing alternative homozygotes. For our *CPS1* and *OTC* PCR products, nearest neighbor analysis predicts no T_m difference between homozygous T/T and A/A amplicons because of nearest neighbor symmetry. However, for both gene targets, the observed mean T_m values are different between homozygote groups, even without calibration. Observed T_m values were 5°C to 7°C higher than theoretical T_m values, caused in part by the presence of LCGreen Plus dye that stabilizes double-stranded DNA hybrids. A nonparametric Mann–Whitney U-test of the uncalibrated and calibrated mean T_m values for the alternate homozygotes indicated significant differences ($P < 0.001$) for all six variants. The T_m variation was lower by 37–90% after calibration. This reduction in variation after calibration is reflected in the T_m ranges for the *CPS1* and *PAH* small amplicons that exhibit overlapping homozygous peaks before calibration that separate into two groups post-calibration (Figures 1 and 2). The single variant where the homozygous alleles were not resolved by calibration was rs1869458 in the candidate gene *BX647987*. For this variant, small T_m differences prevented resolution of the G/G and C/C genotypes (Figure 2). Calibration using both high and low calibrators showed better genotype resolution than using only one calibrator (data not shown).

Excluding the unresolved variant of *BX647987*, genotyping success rates for homozygotes were predicted from the T_m separation and variation at each locus (see Calibration algorithm and genotype prediction section in Materials and methods section). We then determined the observed genotyping error rates by calculating the difference from the measured T_m of each sample to the mean T_m value of either homozygote population within each plate. Since the genotypes of all samples were predetermined by probe-based methods and/or sequencing, we could determine whether each sample's T_m was closer to either the correct or incorrect homozygous group. If the sample's T_m was closer to the mean T_m of the correct group, genotyping was successful. Excluding *BX647987*, homozygous genotyping success ranged from 88.3–91.8% before calibration and improved to 99.2–100% after calibration (Table 4).

To explore the effects of amplicon size on the ability to genotype a base-pair neutral variant, we designed 42-, 50- and 86-bp amplicons that targeted the *CHK2* variant rs9608698. As seen in Figure 3, the separation between the genotypes decreases as the size of the amplicon increases from 42 bp to 86 bp. Derivative melting curves for the 50-bp amplicon are not shown. The results for all three amplicons are summarized in Table 3. The observed mean T_m shifts between G/G and C/C homozygotes in the

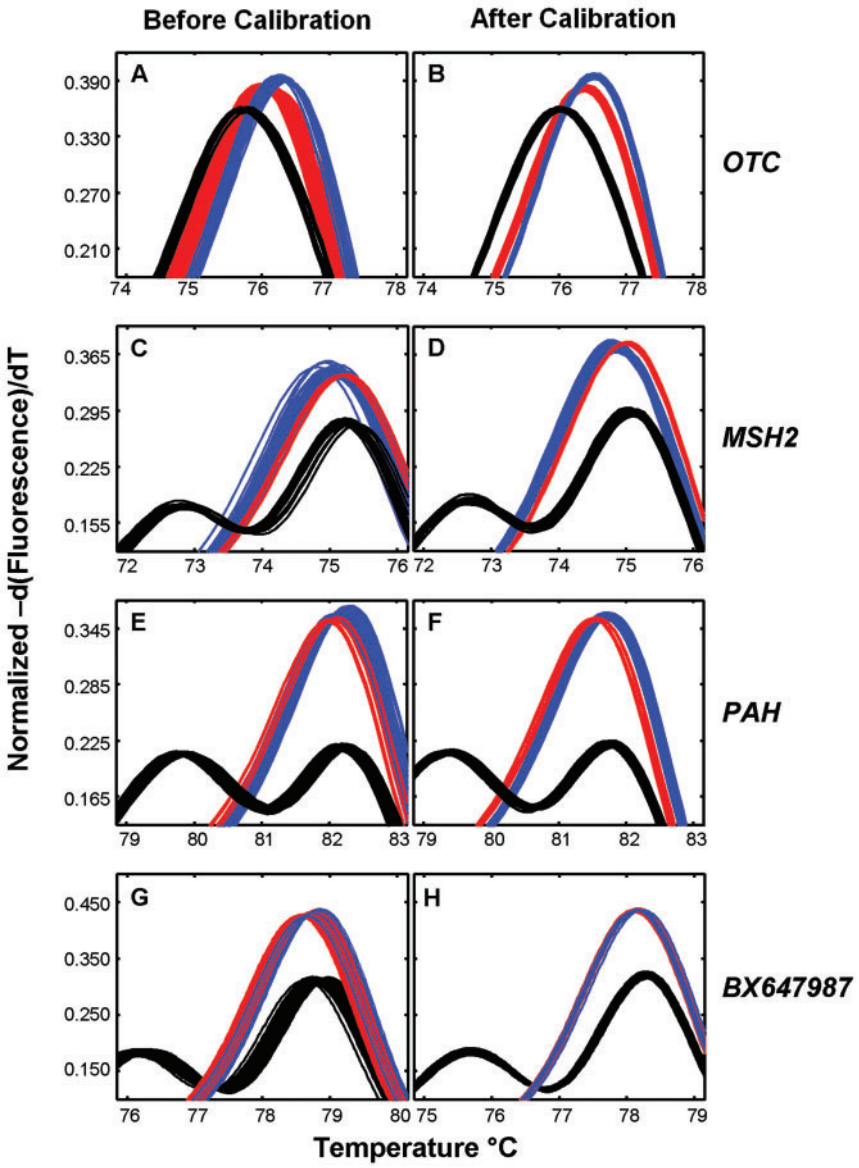

Figure 2. Calibration improves small amplicon genotyping of base-pair neutral changes. The apexes of the derivative melting peaks are shown, before (left panels) and after calibration (right panels), for the *OTC* (**A** and **B**), *MSH2* (**C** and **D**), *PAH* (**E** and **F**) and *BX647987* (**G** and **H**) SNPs. For *OTC*, the A/A genotypes are shown in blue, T/T genotypes in red and A/T heterozygotes in black. For *MSH2*, *PAH* and *BX647987* the C/C homozygotes are shown in blue, G/G in red and C/G heterozygotes in black.

42-bp, 50-bp and 86-bp amplicons were 0.6°C, 0.5°C and 0.3°C, respectively, both before and after calibration.

DISCUSSION

Even in an era of high-throughput sequencing, there is still a need for better genotyping and scanning methods. High-resolution melting allows for simple and inexpensive scanning of full exons for sequence variants (19,20). Small amplicon melting is a genotyping method that complements scanning (21) and is attractive for several reasons. First, the method is homogeneous (closed-tube). Second, T_m alterations from base changes are usually greater with smaller amplicons compared with larger amplicons. This difference is because length is inversely correlated with the magnitude of T_m shift resulting from the base-pair change. Third, the sequence being genotyped is limited to the single base or few bases between the 3′ termini of the forward and reverse primers, limiting the possibility of interference from another variant. Fourth, its greatest appeal may be in its ease and low cost of both reagents and development relative to probe-based genotyping methods (10). Small amplicons generally require minimal optimization to produce robust, clean products that have simple melting transitions.

Several factors can confound genotyping by small amplicon melting. One key factor is inter-sample temperature variation due to differences in the physical

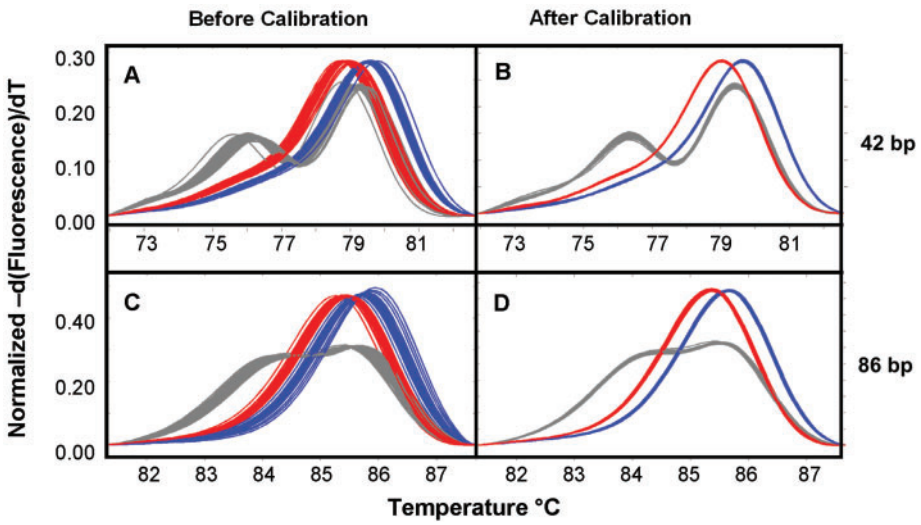

Figure 3. Effects of amplicon size on genotyping the *CHK2* gene. Derivative melting curves for the 42-bp amplicon (**A** and **B**) and the 86-bp amplicon (**C** and **D**) are presented before (left panels) and after (right panels) calibration. The peaks for the low and high T_m calibrators are not shown. The SNP rs9608698 (G>C) from 89 human DNA samples was amplified and analyzed by high-resolution melting. Dual-peaked heterozygotes (gray) are easy to identify without calibration. The G/G and C/C homozygotes in both products are highlighted in red and blue, respectively.

and chemical components of the system. Inter-sample variation appears dependent on the type of melting instrument and reaction vessels; microtiter plate-based systems displayed more variability than capillary-based systems (9). Differences in extraction kit chemistry can also influence the melting curves of small amplicons by changing the ionic strength (16).

We utilized the dual internal oligonucleotide T_m control approach reported by Liew *et al.* (15) and Seipp *et al.* (16) to minimize inter-sample T_m variation. The calibration system we describe herein was able to resolve the homozygotes in five of the six base-pair neutral variants examined that are the most challenging. In fact, the two variants with symmetric nearest neighbors in *CPS1* and *OTC* were predicted by current thermodynamic models to have identical T_m values. Calibration reduced variation within each allele group thereby improving genotyping accuracy. The single variant that was not successfully genotyped by calibration was the C>G variant in *BX647987* rs1869458. The failure to discriminate between the G/G versus C/C homozygotes in this 58-bp amplicon appears to be due to the minimal mean T_m differences of 0.09°C before and 0.02°C between these homozygotes before and after calibration, respectively. These small observed mean delta T_m are consistent with the predicted delta T_m of 0.04°C, and are too small to detect given the 0.079–0.092°C and 0.029–0.036°C standard deviations observed before and after calibration, respectively.

We investigated the impact of fragment length on the CHK2 assay. While the C/C and G/G homozygotes for CHK2 were resolved with 42-, 50- and 86-bp amplicons, the observed delta T_m between the homozygous alleles decreased (~0.6°C, 0.5°C and 0.3°C) as the amplicon size increased (Table 3). This reduced T_m separation is also seen in the derivative melting curves of the heterozygotes that show more distinct peaks in the 42-bp fragment compared to that of the 86-bp fragment (Figure 3).

Internal oligonucleotide calibrators decrease genotyping errors caused by multiple physical and chemistry factors (13–16). Several sporadic samples in our data set displayed T_m far away from other samples of the same genotype before calibration that subsequently grouped within their respective genotype after calibration (e.g. gray heterozygote in Figure 3A versus 3B).

Our calibrators did not adversely affect the eight amplicon melting assays described here with respect to amplification robustness and specificity. We also tested these calibrators with other assays, some in multiplex, and have not observed deleterious consequences (ref. 21 and unpublished data). In theory, internal oligonucleotide calibrators may adversely influence PCR of some targets by either reducing amplification efficiency and/or generating undesired products. Reduction of amplification efficiency may occur if, for example, one of the calibrators can hybridize within the target sequences. The generation of undesired products could occur in two scenarios. In the first case, loss of the 3′ blocks on the calibrators may allow the oligonucleotides to act as primers for undesirable amplification. In the second case, sufficient homology between a primer and one of the calibrator strands may allow primer-to-calibrator products to be made. We have tested calibrators lacking the 3′ blocks and found that high levels of these unblocked oligonucleotides generated additional extension products and melting peaks that obscured analyses of the desired calibrator and target amplicon peaks (data not shown). Decreasing calibrator concentrations or using different calibrators may allow success in cases of initial failure.

In conclusion, internal oligonucleotide calibrators are an attractive and effective way to improve genotyping by amplicon melting. The method is homogenous and simple. Accumulated evidence indicates that calibrators will counter inter-sample variation caused by a variety of physical and chemistry factors. Calibrated small amplicon

Table 3. T_m values of small amplicon homozygotes before and after calibration

Gene	PCR target information			T_m							N^b
	PCR Amplicon Size (bp)	Genotype	Symmetric nearest neighbors?	Predicted[a]	Before calibration			After calibration			
					Average	SD	Range	Average	SD	Range	
CPS1	51	T/T	Yes	72.03	78.28	0.051	78.15–78.39	78.61	0.031	78.55–78.67	32
		A/A	Yes	72.03	78.15	0.049	78.08–78.22	78.46	0.024	78.43–78.50	14
OTC	68	T/T	Yes	70.97	76.11	0.063	75.94–76.25	76.37	0.021	76.32–76.42	70
		A/A	Yes	70.97	76.31	0.055	76.31–76.40	76.52	0.023	76.47–76.55	12
MSH2	49	T/T	No	69.12	75.27	–	75.24–75.29	75.43	–	75.42–75.45	4
		A/A	No	68.93	75.10	0.064	75.02–75.23	75.29	0.025	75.29–75.39	70
PAH	59	G/G	No	75.34	82.13	0.056	82.01–82.26	82.71	0.035	82.60–82.78	58
		C/C	No	75.16	81.95	–	81.86–82.07	82.53	–	82.52–82.55	4
CHK2	42	G/G	No	73.86	79.50	0.090	79.39–79.77	79.62	0.016	79.61–79.66	23
		C/C	No	73.55	78.88	0.119	78.66–79.11	79.00	0.012	78.98–79.02	26
CHK2	50	G/G	No	76.78	81.72	0.063	81.64–81.90	81.82	0.020	81.80–81.87	23
		C/C	No	76.54	81.19	0.056	81.08–81.31	81.30	0.028	81.27–81.38	26
CHK2	86	G/G	No	81.48	85.71	0.078	85.57–85.62	85.68	0.013	85.64–85.69	23
		C/C	No	81.37	85.41	0.060	85.23–85.50	85.38	0.018	85.35–85.41	26
BX647987	58	G/G	No	71.89	78.76	0.079	78.63–78.84	78.14	0.036	78.08–78.22	12
		C/C	No	71.85	78.67	0.092	78.51–78.82	78.12	0.029	78.08–78.17	30

[a]The theoretical T_m values were calculated by inputting the entire sequence of each small amplicon into a program, which utilizes nearest neighbor parameters (11,18) and is available from Idaho Technology, Inc.
[b]The number (n) of samples tested for each genotype was based the sample set surveyed and this may influence the observed mean T_m values.

Table 4. Homozygous genotyping accuracy before and after calibration

Gene/Site	Amplicon Size (bp)	Base change	Nearest neighbor symmetric	Accuracy		n
				Before calibration (%)	After calibration (%)	
CPS1	51	A/T	Yes	91.8	100	184
OTC	68	A/T	Yes	88.4	99.7	328
MSH2	48	A/T	No	91.6	100	296
PAH	59	C/G	No	88.3	99.2	248
CHK2	42	C/G	No	100	100	49
BX647987	58	C/G	No	69	64.3	42

genotyping provides, for the first time, a resolution high enough to accurately genotype base-pair neutral variants, even ones predicted to have identical T_m by current nearest neighbor models. These findings indicate that further investigation of current thermodynamic nearest neighbor models for predicting DNA duplex stabilities is warranted.

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