Simultaneous mutation scanning and genotyping by high-resolution DNA melting analysis

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This protocol permits the simultaneous mutation scanning and genotyping of PCR products by high-resolution DNA melting analysis. This is achieved using asymmetric PCR performed in the presence of a saturating fluorescent DNA dye and unlabeled oligonucleotide probes. Fluorescent melting curves of both PCR amplicons and amplicon–probe duplexes are analyzed. The shape of the PCR amplicon melting transition reveals the presence of heterozygotes, whereas specific genotyping is enabled by melting of the unlabeled probe–amplicon duplex. Unbiased hierarchal clustering of melting transitions automatically groups different sequence variants; this allows common variants to be easily recognized and genotyped. This technique may be used in both laboratory research and clinical settings to study single-nucleotide polymorphisms and small insertions and deletions, and to diagnose associated genetic disorders. High-resolution melting analysis accomplishes simultaneous gene scanning and mutation genotyping in a fraction of the time required when using traditional methods, while maintaining a closed-tube environment. The PCR requires <30 min (capillaries) or 1.5 h (96- or 384-well plates) and melting acquisition takes 1–2 min per capillary or 5 min per plate.

INTRODUCTION

The thermal stability of a PCR product is determined by its base sequence[1]. When the PCR product sequence is altered, duplex stability is changed, leading to different melting behavior. When the change is homozygous, a shift in melting temperature is usually observed[2]. When the change is heterozygous, four duplexes are formed following PCR: two heteroduplexes and two homoduplexes. Each duplex will have differing stabilities, the sum of which can be observed by high-resolution melting analysis (see **Fig. 1**), enabling sequence variations to be detected. During high-resolution melting analysis, melting curves are produced using dyes that fluoresce in the presence of double-stranded DNA (dsDNA) and specialized instruments designed to monitor fluorescence during heating; as the temperature increases, the fluorescence decreases, producing a characteristic melting profile. This melting data can be analyzed to detect sequence variations such as single-nucleotide polymorphisms (SNPs) and small insertions and deletions. In addition, known sequence variants may be genotyped by high-resolution melting analysis using unlabeled oligonucleotide probes. These probes are designed to anneal to either wild-type or mutant sequences and produce melting curves that identify each genotype. Because probe–amplicon duplexes melt at lower temperatures than amplicon duplexes, both PCR product and probe transitions can be analyzed from one melting curve. To maintain a closed-tube environment, these probes are added before PCR and include a 3′ blocker to prevent extension during thermal cycling, and asymmetric PCR is performed to overproduce one strand to which the probe anneals (see **Fig. 2**).

This protocol combines both aspects of high-resolution melting—gene scanning by melting of PCR amplicons and genotyping with unlabeled oligonucleotide probes—into a single closed-tube procedure. High-resolution melting analysis of PCR amplicons is a powerful, homogeneous method for detecting a wide range of mutations at any position within the amplicon[3], with unique advantages over conventional methods. As a closed-tube system, it differs from other gene scanning methods such as single-strand conformational polymorphism analysis[4], denaturing gradient gel electrophoresis[5], heteroduplex analysis[6], denaturing high-performance liquid chromatography[7] or temperature gradient capillary electrophoresis[8]. Each of these methods requires application of the PCR products onto a gel or other matrix to separate and detect the heteroduplexes. In contrast, closed-tube systems eliminate the need for automation, greatly decrease the risk of laboratory contamination from open PCR product and significantly reduce analysis time; melting acquisition and analysis can be performed in less than 10 min after PCR. Conventional closed-tube genotyping techniques require fluorescently labeled probes, which are costly and capable of detecting only a single allele[9]. In contrast, genotyping by

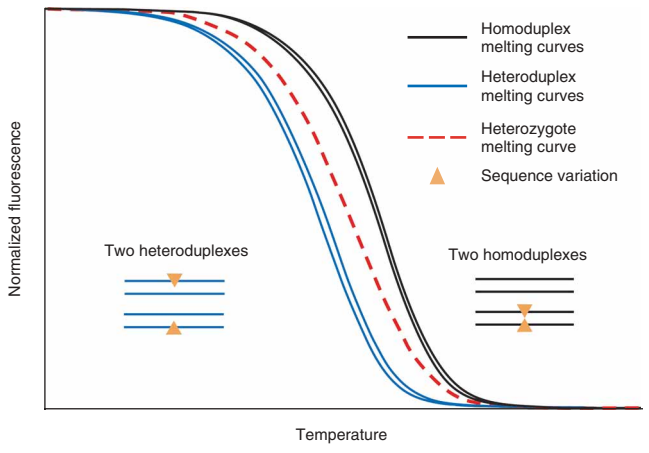

Figure 1 | Melting analysis of heterozygous DNA. After PCR, four species of duplexes are formed from heterozygotes: two homoduplexes and two heteroduplexes. The black lines show melting profiles of the homoduplexes. The blue lines show melting profiles of the heteroduplexes. Instability of heteroduplexes results in a lower T_m than homoduplexes. As heterozygotes contain both heteroduplex and homoduplex species, the heterozygote melting curve is a combination of their melting profiles.

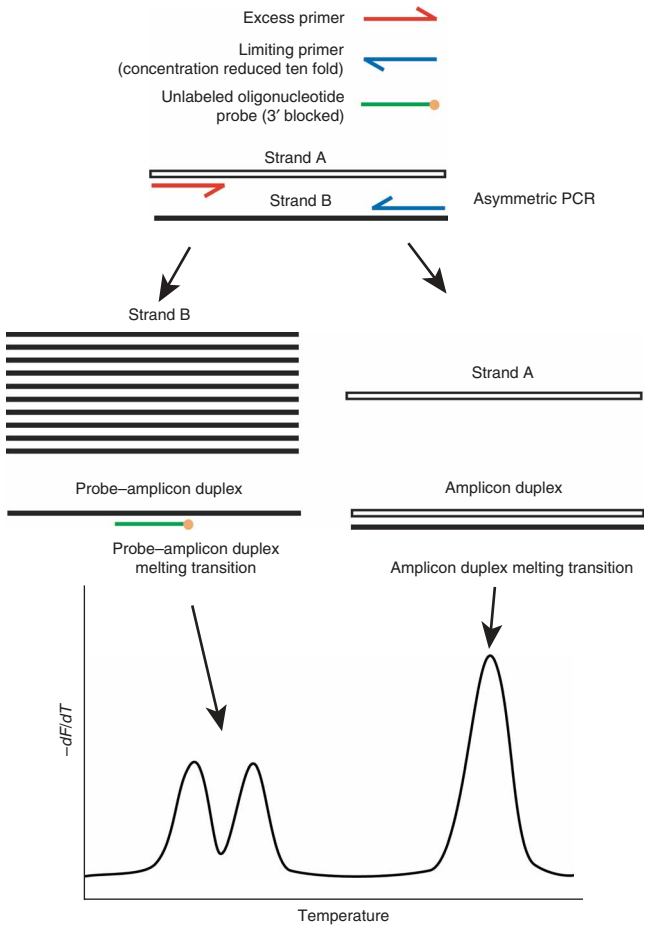

Figure 2 | Diagram of asymmetric PCR in the presence of an unlabeled probe. Excess primer (red) is complimentary to Strand A and produces excess copies of Strand B. The limiting primer (blue) is complimentary to Strand B and produces limited copies of Strand A. Excess copies of strand B increases visibility of the probe-amplicon duplex melting transition. This melting transition is visible on the same melting profile as the amplicon melting transition, enabling simultaneous variant scanning and genotyping. Both melting transitions appear as peaks on a derivative plot, which displays the negative derivative of the normalized fluorescence with respect to temperature ($-dF/dT$) against temperature.

high-resolution melting does not require expensive fluorescent labels and a single unlabeled probe can detect multiple alleles. Additionally, more than one probe may be used to simultaneously genotype sequence variations in different regions of the PCR amplicon[10].

The power of DNA melting analysis depends on the instrument resolution[11], the dsDNA dye[12] and the purity of the PCR product. Historically, DNA melting methods used absorbance to monitor duplex separation over hours in conventional UV spectrophotometers[13]. Over the past decade, rapid fluorescent techniques have been introduced to monitor PCR product[1] or probe[14] melting on real-time PCR instruments. High-resolution DNA melting analysis with fluorescence is a recent development[12,15] allowing greater discrimination between homozygotes and sensitive detection of heterozygotes. The functional characteristics of instruments (i.e., points per °C and signal-to-noise ratio) required for high-resolution melting analysis have been described and compared previously[11]. Fluorescent dsDNA dyes are commonly used to monitor product formation in real-time PCR[16] and to characterize PCR products[1]. However, conventional dyes, including SYBR Green I, have several limitations in permitting the detection of small sequence variations[12,15]. In contrast, the high-resolution dye, LCGreen Plus, used in this protocol, allows the identification of both heterozygous and homozygous SNPs[12], making PCR product scanning and unlabeled probe genotyping possible. Efficient PCR amplification is also necessary for accurate results, and hot-start techniques are often used to increase the purity of the PCR product.

Gene scanning and genotyping by high-resolution melting are performed by simple analysis of a single melting profile. Gene scanning relies on the change in the shape of the amplicon melting transition curve introduced by heterozygous sequence alterations. After background subtraction, normalization and unbiased hierarchal clustering, sequence variants are identified as groups that exhibit similar melting profiles. These groups can then be genotyped for known sequence variations by examining probe-melting transitions. A single base-pair change within the probe region causes a significant shift in the melting temperature (T_m) of the probe–amplicon duplex, making it easy to identify specific genotypes. Using this technique, both homozygous and heterozygous sequence variants can be reliably differentiated[3,10,15]. Even multiple variants in close proximity to each other are distinguishable, for example, all genotypes of the neighboring HbS and HbC single-base variants in β-globin[12,15]. In PCR products up to 1,000 bp, high-resolution melting has been used to detect heterozygous SNPs with a sensitivity and specificity of 97% and 99%, respectively[3]. With well-optimized PCR, most sequencing becomes unnecessary because sequence variants either cluster as wild type by PCR product melting or are specifically identified by unlabeled probes. Rare or new variants that are not identified can be subsequently characterized by sequencing because melting is not destructive. High-resolution melting has been applied to a variety of applications including SNP typing[17], unlabeled probe genotyping[18], HLA matching[19] and scanning for mutations within the medium-chain acyl-CoA dehydrogenase[20] and *c-kit*[21] genes. It can be applied in any research or clinical setting for the study of SNPs and small deletions/insertions as well as diagnosis of genetic disorders associated with these mutations.

Experimental Design

Sample format for high-resolution melting analysis

PCR and DNA melting can be performed either in capillaries or plates (96- or 384-well). Best results are obtained in instruments specifically designed for high-resolution melting[11]. The main difference between capillary and plate formats is the throughput. Using capillaries, up to 32 samples are amplified at a time, and each capillary is analyzed sequentially. For high-throughput applications, 96 or 384-well plate format is useful.

DNA preparation

Genomic DNA can be isolated and purified using any method ranging from conventional phenol/chloroform extraction and ethanol precipitation[22] to modern automated solid-phase-binding methods[23]. However, all DNA samples that will be compared should be dissolved in the same buffer solution because different buffers will affect the melting temperature. Variable concentrations of salt and ethylenediaminetetraacetic acid as well as additives commonly used in PCR such as glycerol, dimethyl sulfoxide and betaine are compatible with this technique as long as they are consistent between samples. Up to a tenfold variation in initial

DNA concentration is acceptable because an optimized PCR tends to equalize product concentrations and T_m is only weakly dependent on DNA concentration[15]. For example, DNA extracted from dried blood spots has been used successfully for mutation scanning.

PCR optimization

For best results, the PCR should be optimized to produce specific product with adequate yield by varying the annealing temperature and/or the magnesium ion concentration ($[Mg^{2+}]$). Perform PCR using 2.0, 2.5 and 3.0 mM final $[Mg^{2+}]$ to find the best conditions. Also, try adjusting the PCR annealing temperature between 50 and 65 °C. Run an agarose gel of the product to ensure that it is the appropriate size and is free from extraneous product. If desired, the product may be sequenced to further confirm PCR specificity. Both $[Mg^{2+}]$ and annealing temperature can be quickly optimized with the use of gradients. With capillaries, perform PCR at different $[Mg^{2+}]$, repeating at several annealing temperatures. For 96- or 384-well plates, an annealing temperature gradient across the plate allows simultaneous optimization of both $[Mg^{2+}]$ and annealing temperature. Compare purity and yield of these products on an agarose gel to determine the most appropriate conditions. Select the conditions that yield the purest product; because of the sensitivity of high-resolution melting, purity is more essential than quantity. If necessary, the primer concentrations may also be varied.

Other parameters such as the extension time and number of PCR cycles can also be adjusted during optimization. Asymmetric PCR normally requires 5–10 more cycles than conventional PCR to reach plateau. Also, the presence of probes may inhibit extension if the probe T_m is greater than the PCR extension temperature. In this case, an extension temperature ramp (0.5 °C s^{-1} from 70 to 78 °C), or use of an exonuclease-positive polymerase will increase the PCR efficiency. At the end of temperature cycling, include a denature/renature cycle of heating to 95 °C followed by rapid cooling to at least 55 °C to promote heteroduplex formation and detection; heteroduplexes and unlabeled probes are best detected after rapid cooling[15].

Reference controls

A negative control including all reagents minus DNA may be used to ensure that the PCR products are not the result of carryover contamination of DNA into other reagents. Negative PCR controls are verified and eliminated before normalization is performed. Known reference genotypes are useful in analyzing PCR product melting curves, especially when only a few samples are analyzed. When many samples are analyzed, common genotypes usually display characteristic shapes and appear at an expected frequency. When unlabeled probes are designed to match the sequence variant of interest, positive controls are not necessary within each run (see 3′ oligonucleotide probe below). Replicates can be used to provide an estimate of variation within a genotype, but are not required and are seldom used. A single unknown sample may be scanned and genotyped for mutations using a single known wild type as a reference.

Oligonucleotide primers

Scanning and genotyping is possible with PCR products of 1,000 bp, highly accurate at < 400 bp and optimal when the product size is <200 bp. The T_m of the PCR product should be at least 2–4 °C below the boiling point of water at the altitude where the PCR is performed to allow observation of the entire PCR product melting curve. If necessary, dimethyl sulfoxide, betaine or glycerol may be added to lower the T_m. In order for the probe melting transition to be visible, the PCR must be asymmetric with one primer at a concentration 5–10-fold less than the other primer. Make sure the unlabeled probe is complimentary to the DNA strand formed by the excess primer.

3′-blocked oligonucleotide probe

The unlabeled probe is designed to hybridize to the region of sequence variation to be genotyped. It may be designed to perfectly complement the wild-type sequence or the sequence variant. Matching the sequence variant is especially useful if there are other sequence variations that may also be within the probe region. In this case, only the targeted variant will be perfectly matched and have the highest probe T_m. The wild-type sequence will have one mismatch, whereas other sequence variations will result in two regions of mismatch and further destabilize the probe. Single (see **Fig. 3**) or multiple (see **Fig. 4**) variants are distinguishable under the probe by T_m. Mismatches at the ends and penultimate bases of the probe will not destabilize the duplex as much as more central positions. Longer probes (near 30 bp) produce the best signal, especially with longer PCR products. Owing to differences in length, the melting transitions of the probe–amplicon duplex will not overlap with that of the amplicon duplex; however, the T_m of the oligonucleotide probe should be below 70 °C so that it does not interfere with polymerase extension. Deliberate mismatches or polymorphism masking[24] can be used to decrease the probe T_m, as well as prevent genotyping of inconsequential SNPs that are known to be within the same probe region. Alternatively, probe T_m values greater than 70 °C can be used if an exonuclease positive polymerase is used so that extension is not inhibited[24]. Probes as small as 15 bases or less have been used[18]. The 3′ end of the probe must be blocked to prevent its extension during PCR. Most commonly, 3′-phosphorylation is used. However (depending on the vendor), 3′-phosphorylation may not be complete, leading to additional melting transitions between those of the amplicon and probe–amplicon duplexes. Better blockage has been reported with a 3′-C3 blocker[25]. More than one probe may be used to genotype sequence variations in different regions of the amplicon[10]. Each probe is typically used at a final concentration of 0.5 μM. Multiple probes should be designed so that all alleles are separated by a T_m difference of at least 2 °C to allow easy genotyping of heterozygous genotypes.

Melting rate

Optimal heating rates are 0.1–0.3 °C s^{-1}. Faster heating rates improve heteroduplex detection sensitivity; however, slower heating rates better resolve different homoduplexes[15]. On the single sample HR-1 instrument, the best compromise between quality and throughput is 0.3 °C s^{-1}. On the LightScanner, a melting rate of 0.1 °C s^{-1} provides higher quality data.

Melting data analysis

High-resolution melting instruments come equipped with software that facilitates analysis of probe and amplicon melting transition regions. Scanning and genotyping sequence variants are simple and straightforward. Briefly, a description of these analysis functions is as follows.

Background subtraction and data normalization. Background fluorescence approximates an exponential and is first estimated and eliminated by subtraction. Two temperature intervals are selected

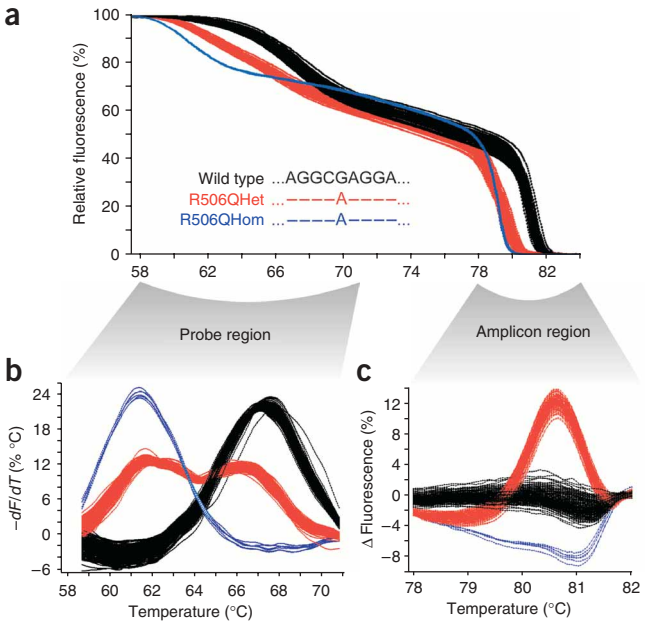

Figure 3 | High resolution melting analysis of the Factor V Leiden SNP. Three genotypes are present in this 384-well plate assay: wild type (black lines), R506Q heterozygote (red lines), and R506Q homozygote (blue lines). (**a**) Probe and amplicon melting transition regions after exponential background subtraction and data normalization. (**b**) Derivative plot of the probe melting region shows the negative derivative of normalized fluorescence with respect to temperature ($-dF/dT$) against temperature. Melting transitions appear as peaks. With the probe designed complimentary to the wild type, this genotype has the highest T_m. Homozygous mutants have a single peak at a lower temperature. Heterozygotes have two subdued peaks at both temperatures. (**c**) Difference plot of the amplicon melting region. Differences in amplicon melting transitions for each genotype are more easily seen. Reprinted with permission from *Clin Chem* **51**, 1770–7 (2005).

within which no melting occurs, one below the melting transition of interest and one above. Within each interval, the slope (derivative) of the background fluorescence is determined using least-squares fitting. The intervals should be as close to the regions of melting as possible without overlapping it, and as narrow as possible while still smoothing any point-wise noise. Using these two slopes and the midpoint temperatures of the two intervals, a unique exponential function is calculated. Next, the exponential background model is subtracted from the raw fluorescence, leaving the background-subtracted fluorescence curve. Although unlabeled probe genotyping can be performed by directly taking the derivative without exponential background subtraction, the fluorescent background at low temperatures is high[18] and better results are obtained with background subtraction. Finally, the background-subtracted fluorescence curve is normalized between 0% and 100% by subtracting the minimum fluorescence from all values, dividing by the resultant maximum fluorescence, and multiplying by 100. Normalized plots of both the probe and amplicon transitions (**Figs. 3a** and **4b**) are only possible after appropriate background subtraction. Detailed algorithms are published elsewhere[26].

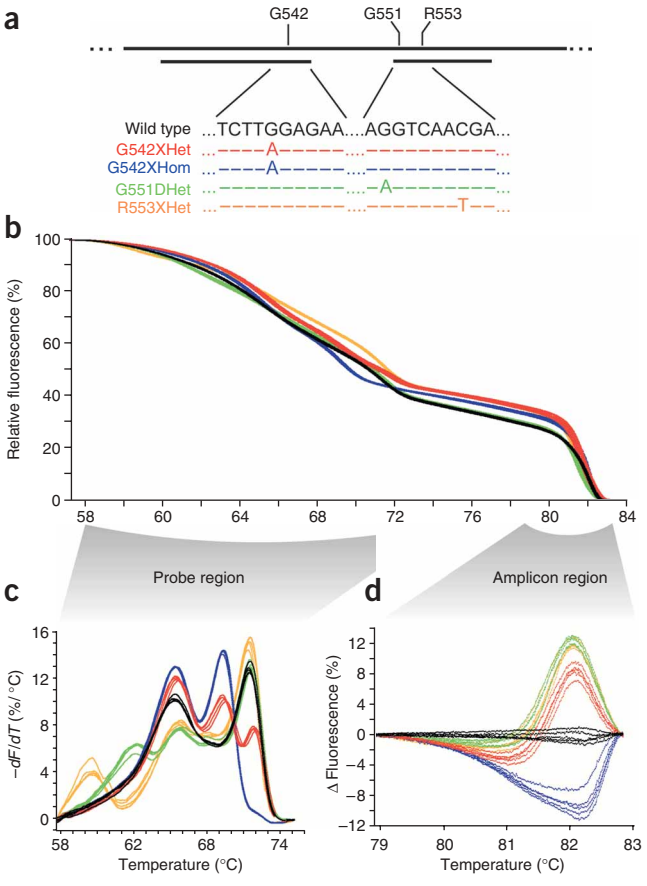

Figure 4 | High resolution melting analysis of exon 11 of the cystic fibrosis transconductance regulator (CFTR) gene. Two probes are used to genotype three mutations (G542X, G551D, R553X). Melting data was obtained using an HR-1. (**a**) Relative position of the two probes and sequence details of each of the five genotypes. (**b**) Probe and amplicon melting transition regions after exponential background subtraction and data normalization. (**c**) Derivative plot of the probe melting region shows the negative derivative of fluorescence with respect to temperature ($-dF/dT$) against temperature. Unique peak patterns reveal the five different genotypes. (**d**) Difference plot of the amplicon melting region. The melting curve shape of each variant genotype is distinctly different from the wild type. Reprinted with permission from *Clin Chem* **51**, 1770–7 (2005).

Curve overlay. Minor temperature errors or buffer differences between samples can be corrected by "temperature shifting" each curve so that they are overlaid across a portion of the curve. This allows better clustering of samples for heterozygote scanning. An interval is selected where all curves are similar, usually at high temperature and low fluorescence (e.g., between 2% and 5% normalized fluorescence). The normalized fluorescence values within this interval are extracted and temperature versus normalized fluorescence functions obtained. The average temperature for each function is calculated. The average temperature difference between each curve and a randomly selected standard curve is subtracted from the independent variable (temperature) for each curve. This equalizes the average temperature of all melting curves to that of the standard curve in the fluorescence range selected. It should be noted, however, that homozygous variants are best detected without temperature shifting; so amplicon melting data should be analyzed both with and without temperature shifting if homozygous variants are possible.

Variant clustering. Clustering of melting curves identifies and groups variants, and is automatically accomplished by standard,

unbiased, hierarchical clustering using the difference metrics described below. A clustering hierarchy is determined by iteratively replacing the closest two curves from the previous stage of iteration by their weighted average, beginning with the original melting curves and ending when only one curve remains. The weight of any curve is the sum of the weights of the two curves, which are averaged; therefore, it is the total number of original curves that have been combined to form the new curve. The distance between curves is determined by various standard metrics, typically their mean absolute difference, maximum absolute difference or mean-square difference. The hierarchy level that best discriminates the desired groups is determined. This cluster level may be identified automatically by finding the maximum ratio of the distances between clusters joined at successive levels. In this case, instead of the weighted distance between clusters, the distance between the closest pair of curves taken from different clusters is used.

Difference plots. Scanning for variants relies on changes in the melting curve shape. These changes are most apparent on differences plots of normalized and overlaid curves. The wild-type or most populous cluster is selected as a reference. The fluorescence values of each curve are subtracted from the average reference values. If the reference curves are the most stable products (have the highest fluorescence at a fixed temperature), the wild-type curves form a baseline, with other groups displayed as positive differences. Optionally, the differences in regions of sharp and gradual transitions may be balanced by approximating the orthogonal difference between curves. This is carried out by multiplying the differences computed in the previous step by a slope-dependent factor, which increases to a value of 1 in flat regions. This factor is the inverse square root of 1 plus the average of the squares of the slopes of the two curves at the temperature of interest.

Negative first derivative plots. Probe T_m values appear as maxima on negative first derivative plots of normalized melting data, making it easy to genotype sequence variants. The negative first derivative is determined by Solvitsky–Golay polynomial estimation[27].

MATERIALS

REAGENTS

- 10× PCR buffer: 30 mM $MgCl_2$, 2.5 mg ml^{-1} bovine serum albumin (BSA), 500 mM Tris pH 8.3 (Idaho Technology Inc., cat. no. 1770)
- TaqStart antibody (1,100 ng μl^{-1}; Clontech, cat. no. 639250)
- KlenTaq1 (25 U μl^{-1}; AB Peptides)
- Enzyme diluent: 10 mM Tris, pH 8.3, 2.5 mg ml^{-1} bovine serum albumin (Idaho Technology Inc., cat. no. 1773)
- 10× dNTP mix: 2 mM dATP, dCTP, dGTP, dTTP (Idaho Technology, cat. no. 1774).
- 10× LCGreen Plus (Idaho Technology Inc., cat. no. BCH-ASY-0005) Alternatively, a commercial, readymade 2.5× master mix including all the above components is available (Idaho Technology Inc., cat. no. HRLS-ASY-0002). ▲ CRITICAL Use of LCGreen dyes is essential. Other fluorescent DNA dyes such as SYBR Green I do not detect heteroduplexes well[12]. LCGreen I can be used on the HR-1 instrument, but not on the LightScanner, where the brighter LCGreen Plus is necessary.
- Genomic DNA (50 ng μl^{-1}; see Experimental Design regarding the preparation of DNA) ▲ CRITICAL For best results, all human genomic DNA samples compared should be prepared by the same method and the concentration adjusted to an $A_{260} = 1.0$ (~50 ng μl^{-1}). For DNA from other sources (e.g., bacteria, plasmids), use a concentration of about 10^5 copies per µl.
- 10× Reverse direction oligonucleotide primer (5 µM; see Experimental Design)
- 10× Forward direction oligonucleotide primer (0.5 µM; see Experimental Design)
- 10× 3′-blocked forward direction oligonucleotide probe (5 µM; see Experimental Design). All oligonucleotides were obtained from the University of Utah core synthesis facility and were synthesized by standard phoshoramidite chemistry.

EQUIPMENT

- LC Carousel Centrifuge (Roche, cat. no. 03709507001)
- LightCycler Capillaries (Roche, cat. no. 11909339001)
- LightCycler 1.2-2.0 (Roche, cat. no. 04484495001)
- LightCycler Centrifuge Adapters (Roche, cat. no. 11909312001)
- HR-1 (Idaho Technology Inc., cat. no. HR01-ASY-0001)
- 96- or 384-well plates (Bio-Rad, cat. nos. 96-well: HSP-9665; 384-well: HSP-3865)
- Peltier Thermal cycler (Bio-Rad, cat. no. PTC-0200)
- LightScanner (Idaho Technology Inc., cat. nos. 96-well: LSC9-ASY-0011; 384-well: LSC9-ASY-0001) ▲ CRITICAL The accuracy of scanning and genotyping by PCR product melting analysis is dependant on the instrument resolution[11]. Instruments specifically designed for high-resolution melting (i.e., HR-1 and LightScanner) are used here and produce the best results. Genotyping with unlabeled probes alone can be performed on lower-resolution instruments such as the LightCycler and LightTyper[18]; however, similar results for this protocol cannot be expected from lower-resolution instruments, including conventional real-time instruments.

REAGENT SETUP

10× Taq polymerase/antibody For a 100 µl volume of this solution, combine 8 µl TaqStart antibody, 16 µl KlenTaq1 and 76 µl enzyme diluent.

PROCEDURE

PCR amplification of target DNA ● TIMING 30 min to 2.5 h

1| Prepare a PCR master mix for each sample to be amplified, as follows:

Reagent	Volume, each reaction (µl)
10× PCR buffer	1
10× *Taq* polymerase/antibody	1
10× dNTPs	1
10× LCGreen Plus	1
10× Forward primer	1
10× Reverse primer	1
10× Probe	1
H_2O	2
Final volume	9

Alternatively, use the commercially available master mix:

Reagent	Volume, each reaction (µl)
2.5× LightScanner PCR mix	4
10× forward primer	1
10× reverse primer	1
10× probe	1
H_2O	2
Final volume	9

▲ CRITICAL STEP For experiments with only a few samples, a known wild type should be run in parallel. With larger scale experiments, the most populous genotype may be used as a reference. Also, the [Mg^{2+}] in the PCR buffer for all samples may need to be adjusted for optimal PCR specificity (see Experimental Design). Buffers with different [Mg^{2+}] are available from the same vendor.

2| Perform PCR in a capillary format using the LightCycler (option A; for smaller scale PCR), or in a 96- or 384-well plate format in a plate thermal cycler (option B; for high-throughput processing).

(A) Smaller scale capillary format using a LightCycler ● TIMING 30–45 min

(i) Aliquot 9 µl PCR master mix into an appropriate number of LightCycler capillaries, one for each sample to be tested.
(ii) Add 1 µl genomic DNA to each capillary.
(iii) Cap each capillary.
(iv) Centrifuge samples using LightCycler Centrifuge Adapters or the LC Carousel Centrifuge.
(v) Place LightCycler Sample Carousel in LightCycler.
(vi) Perform PCR with appropriate cycling conditions, as follows:

	Cycle	Temperature (°C)	Duration (s)
	Denature	95	15
35–60 cycles	Denature	95	0
	Annealing	50–65	0
	Extension	74	5
	Denature	95	0
	Renature	55	0

▲ CRITICAL STEP PCR product yield and purity greatly influence the quality of high-resolution melting data; thus, cycling conditions must be optimized for each target (see the Experimental Design section).

(B) Larger scale 96- or 384-well plate format using a plate thermal cycler ● TIMING 1.5–2.5 h

(i) Aliquot 9 µl PCR master mix into an appropriate number of wells of a 96- or 384-well plate, one for each sample to be tested.
(ii) Add 1 µl genomic DNA to each well.
(iii) Add 10–20 µl mineral oil to each well.
(iv) Centrifuge plate at 20–25 °C for 2 min at 1,500*g*.
(v) Place 96- or 384-well plate in thermal cycler.
(vi) Perform PCR with appropriate cycling conditions, as follows:

	Cycle	Temperature (°C)	Duration
	Denature	95	2 min
35–60 cycles	Denature	95	30 s
	Annealing	50–65	10 s
	Extension	74	30 s
	Denature	95	30 s
	Renature	55	Hold

▲ CRITICAL STEP PCR product yield and purity greatly influence the quality of high-resolution melting data; thus, cycling conditions must be optimized for each target (see Experimental Design section).

■ PAUSE POINT PCR products may be stored at 20–25 °C overnight or at 2–4 °C for up to a week.

Melting acquisition ● TIMING 2 min per capillary or 10 min per plate

3| Acquire melting data from the LightCycler capillaries using an HR-1 (option A; for smaller scale analysis), or from the 96- or 384-well plate using a LightScanner (option B; for high-throughput analysis).

(A) Melting acquisition from LightCycler capillaries using an HR-1 ● TIMING **2 min per capillary**

(i) Insert a capillary into the HR-1.

(ii) Melt sample from 50 to 90 °C at a rate of 0.3 °C s^{-1}.

(iii) Repeat Steps (i) and (ii) for each sample.

(B) Melting acquisition from 96- or 384-well plate using a LightScanner ● TIMING **10 min per plate**

(i) Briefly spin down plate at 1,500*g*.

(ii) Insert 96- or 384-well plate into the LightScanner.

(iii) Melt samples from 50 to 90 °C at a rate of 0.1 °C s^{-1}.

Melting analysis ● TIMING **2–10 min**

4| Starting with the original melting curve data, remove the background fluorescence by fitting a decreasing exponential to the slope of the curve in regions where no melting occurs (see Experimental Design for details).

5| Normalize all plots between 0% and 100% fluorescence (see Experimental Design for details).

6| To perform unlabeled probe genotyping, use the background-subtracted, normalized data and analyze the probe region by taking the negative first derivative (see Experimental Design for details; see **Figs. 3b** and **4c**). Different alleles appear as different peaks.

7| To scan the amplicon for sequence variants, analyze the shape of the amplicon melting transition (using the background-subtracted, normalized data) with and without curve overlay (see Experimental Design for details).
▲ CRITICAL STEP Curve overlay by temperature shifting corrects for minor sample differences (ionic strength, spatial or temporal temperature variation) and increases the ability to distinguish heterozygotes from homozygotes. However, homozygotes are better resolved without curve overlay.

8| Cluster each genotype by standard, unbiased hierarchal clustering using the difference metrics described above (see Experimental Design for details; see color-coded genotype clusters; **Figs. 3** and **4**).
▲ CRITICAL STEP If homozygous variants are possible, clustering is most effective when performed without curve overlay in Step 7.

9| Display the melting curves as difference plots (see Experimental Design for details; see **Figs. 3c** and **4d**).

● TIMING

Steps 1 and 2: 30 min to 2.5 h
Step 3: 2 min per capillary, 10 min per plate
Steps 4–9: 2–10 min

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reasons	Solution
Extraneous melting transitions or poor curve clustering	Secondary PCR products	Optimize PCR conditions to obtain clean product
	Low PCR yield	Optimize PCR to enhance product yield
	Inconsistent genomic DNA preparation	Ensure that the genomic DNA concentration and buffer is consistent
Amplicon and probe melting transitions not visible or are very small	Probe T_m too high, preventing PCR extension	Redesign probe with lower T_m, use an exonuclease-positive *Taq* or add the probe after PCR
	Amplicon too long	Design primers for shorter amplicon length
	Low PCR yield	Optimize PCR to enhance product yield
PCR product T_m too high	High GC content	Add DMSO, betaine or glycerol to the PCR buffer

ANTICIPATED RESULTS

When PCR is properly optimized, sequence variants will cluster tightly, making it simple to scan a large number of samples for specific alterations. Melting curve profiles generated from PCR products in the presence of unlabeled oligonucleotide probes

have two distinct melting transition regions, corresponding to the amplicon and amplicon–probe duplexes (**Figs. 3** and **4**). The melting transition region with the most dramatic decrease in fluorescence corresponds to the PCR amplicon and occurs at a higher temperature. Sequence variants are distinguishable within this region by a change in melting curve shape and are made more apparent with a difference plot (**Fig. 3c**). At a lower temperature, more subtle melting transitions occur corresponding to the unlabeled oligonucleotide probes. With appropriate background subtraction and conversion to a derivative plot, these probe melting transitions appear as peaks (**Fig. 3b**). The T_m values of these peaks correspond to the alleles present in the sample. The allele that forms the most stable duplex with the probe will have a melting peak at a higher temperature. For example, if a probe is designed identical to the wild type, samples that are homozygous for the wild-type allele will have a single melting peak at a higher temperature (**Fig. 3b**, black lines); samples that are homozygous for a mutated allele will have a single melting peak at a lower temperature (**Fig. 3b**, blue lines); and samples that are heterozygous for both alleles will have two subdued peaks at both temperatures (**Fig. 3b**, red lines). More than one unlabeled probe may also be used to genotype sequence variants in distant regions of the same amplicon (**Fig. 4a,b**). On a derivative plot of the probe region, unique peak patterns reveal each genotype (**Fig. 4c**). Each variant is also distinctly different from the wild type in the amplicon melting region when viewed on a difference plot (**Fig. 4d**).

COMPETING INTERESTS STATEMENT The authors declare competing financial interests (see the HTML version of this article for details).

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