Chapter 2

Genotyping DNA Variants with High-Resolution Melting Analysis

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Abstract

High-resolution melting analysis (HRMA) is a simple, quick, and effective method to scan and screen PCR amplicons for sequence variants. HRMA is a nondestructive closed tube assay; after PCR, DNA melting can directly be performed on the amplified samples without any purification or separation steps. For single SNP genotyping, HRMA is an attractive alternative to Sanger sequencing, restriction enzyme analysis, and hydrolysis probes.

Key words Single nucleotide polymorphism, Variant detection, Melting curve analysis, DNA

1 Introduction

DNA melting is the process where a transition from double-stranded (ds)DNA to single-stranded (ss)DNA occurs by increasing the temperature. The thermal denaturing behavior of dsDNA is dependent on base composition; it describes the manner in which dsDNA undergoes the transition to ssDNA. Not only the GC content but also the nucleotide distribution determines how dsDNA melts. The temperature at which 50% of all dsDNA species have become single stranded is called the melting temperature (T_m). Any sequence variant can lead to a different melting behavior and T_m, which makes it possible to detect these changes by monitoring the melting process. HRMA is usually performed on amplicons in the presence of a saturating fluorescent DNA binding dye such as LC-green Plus. It is important that the dye is saturating, which means that a dye molecule occupies every binding position in the DNA. Classical dyes like SybrGreen are not suitable for HRMA, since they are not used at a saturating concentration.

HRMA is very sensitive for detecting heteroduplexes: re-annealed opposite strands of the two alleles in which there will be one or more mismatches. Heteroduplexes will usually form during PCR in DNA samples that contain heterozygous variants.

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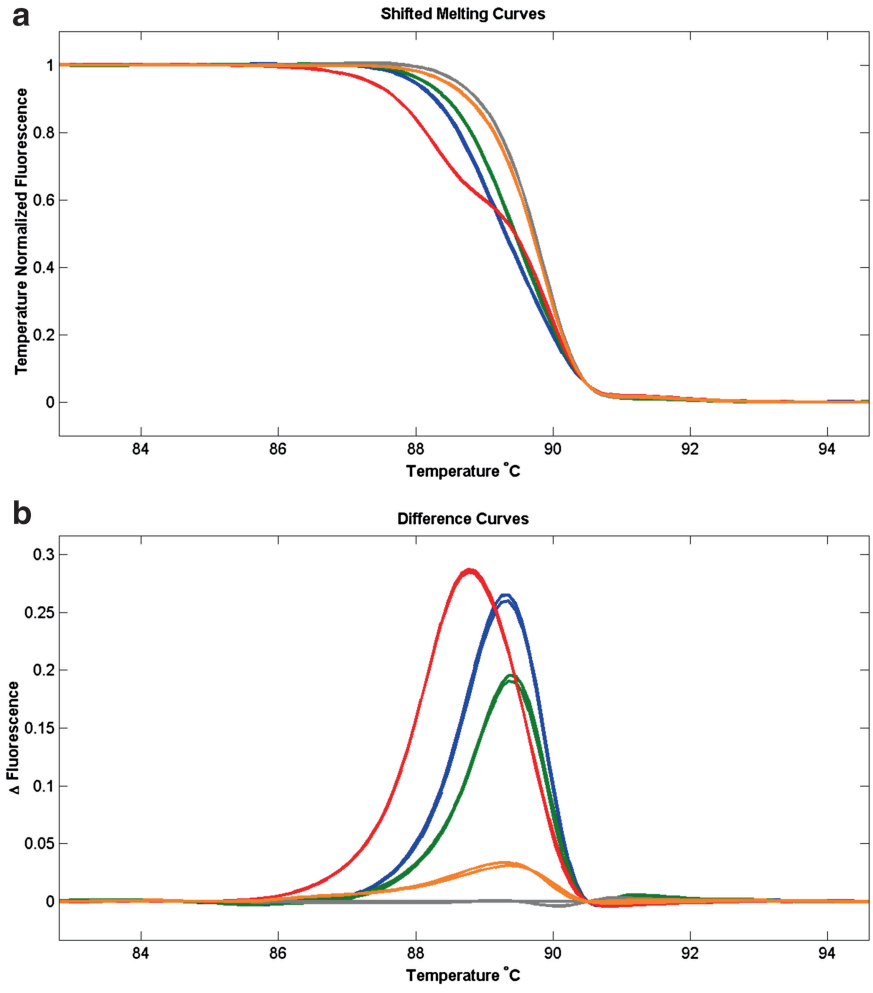

Fig. 1 (**a**) Temperature normalized melting curves, and (**b**) difference curves from DNA samples containing different sequence variants in the same amplicon

For heteroduplex detection, HRMA relies more on the shape of the melting transition then on the T_m [1] (Fig. 1). Heterozygous variants give rise to an altered melting curve and are detected with high sensitivity. In contrast, homozygous variant detection relies more on T_m change and is therefore detected with significantly lower confidence. HRMA sensitivity has also shown to be less sensitive for detecting small insertions and deletions, and these can occasionally be missed. The accuracy of HRMA depends on the instrument, the software, and the fluorescent dye being used [2].

1.1 HRMA: Scanning and Screening

Detection of unknown sequence variants in amplicons is called scanning. When a rare variant is expected in a large number of samples, scanning with HRMA will significantly reduce the

workload when compared to Sanger sequencing. Known mutations can be more efficiently targeted, either by small amplicon melting or unlabeled probe melting. The workflow for scanning and screening is straightforward: after careful optimization of the PCR (*see* Subheading 1.5), samples are amplified in 96- or 384-well plates, either in real-time fashion or on a regular block thermocycler. After PCR, samples are melted and analyzed with appropriate software, which groups melting curves with overlapping profiles. HRMA is a comparative analysis: melting curves of unknowns are compared to those of a control sample which defines the baseline.

1.2 Assay Design

Scanning for unknown coding variants usually involves designing primers that cover the exons of a gene and some flanking intron sequences. Idaho Technologies has developed LightScanner primer design software that is convenient to use. It automatically generates multiple overlapping primer sets from exonic regions, thereby designing amplicons with sizes that are suitable for HRMA. For scanning and screening, amplicon size is important. In general, variants in small amplicons will be detected with greater sensitivity than in larger amplicons. The sensitivity for heterozygous variants in amplicons up to 300 bp is nearly 100% [3] and this is the maximum size recommended for diagnostic purposes. Heterozygous variants in amplicons larger than 400 bp and up to 1000 bp can be detected with a sensitivity higher than 95% [3]. When amplicon size is not important, it is best to keep the size as small as possible.

For the screening of known SNPs, it is efficient to narrow down the region of interest. This can either be done with an unlabeled probe assay or small amplicon PCR.

1.2.1 Unlabeled Probes

Unlabeled probes are convenient to use for SNP typing and the detection of small deletions [4]. In addition to the PCR primers that span a small region of 100–300 bp, a non-fluorescent 3′-blocked oligo of 20–30 nt covering the variant of interest is introduced. The location of the SNP in the probe sequence may vary but will preferably be in the middle. Better differentiation is achieved when the mismatch is in the central portion of the probe [2]. The oligo can be 3′-blocked by either a phosphate group, a dideoxy nucleotide, or an amino C3 or C6 linker. The phosphate group tends to be a less stable modification [5]. It is possible to target multiple SNPs that are in close proximity of each other with a single probe [6]. PCR with unlabeled probes is done in an asymmetric fashion: if the probe was designed on the forward strand, a 1:5–10 ratio of forward to reverse primer is used. This enhances the probe signal. One should first determine the best ratio of forward to reverse primer by testing ratios of 1:5, 1:10, and 1:15. Small amplicons are generally preferred, but in our hands amplicons with sizes of 100–200 bp gave better results with a probe than amplicons smaller than 100 bp.

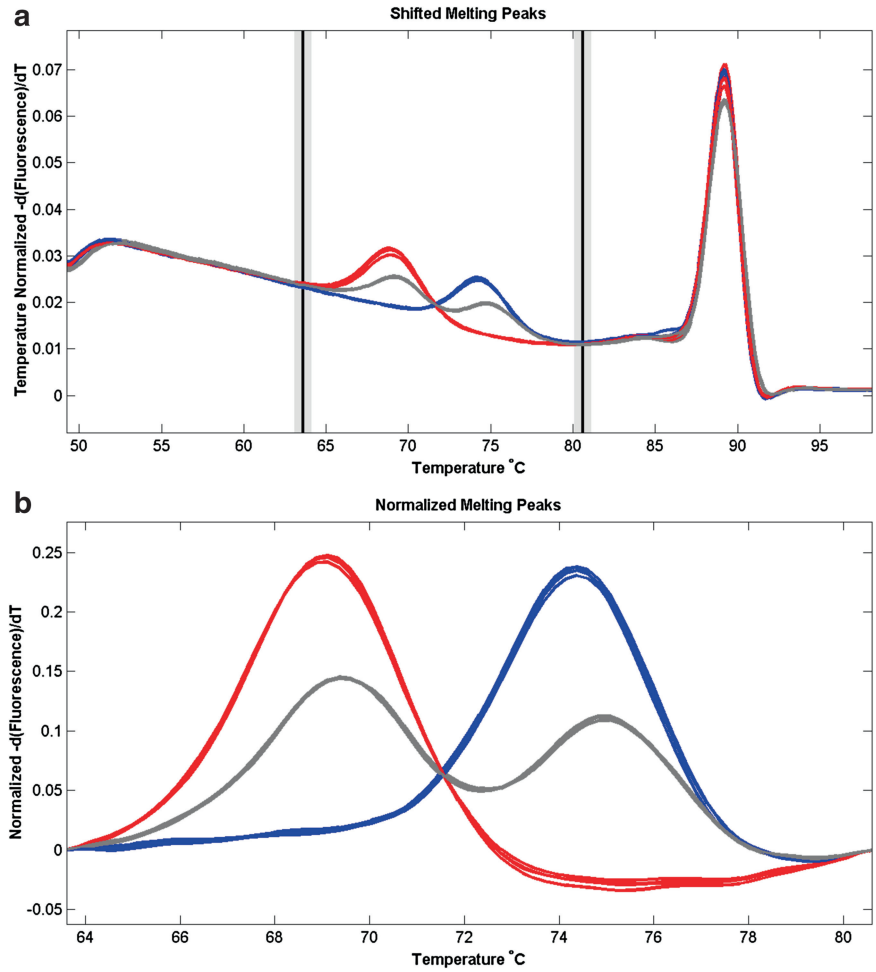

Fig. 2 (**a**) Shifted and (**b**) normalized melting peaks of an unlabeled probe assay targeting a C>G substitution. The probe sequence included the C-variant: homozygous C and thus 100 % match will have the highest T_m (*blue*). *Red* and *gray* are G/G and C/G respectively. In (**a**), both the probe melting curves in the middle and the whole amplicon melting peak at the right are seen

1.2.2 Small Amplicon PCR

For single SNP detection, one can design PCR primers directly before and after the SNP and amplify a fragment ≤50 bp [7]. All heterozygous variants will be easily detected, but resolving homozygous variants can be challenging. Especially when the GC content stays the same (e.g. G/C or A/T variants), the T_m differences will be very small and hard to detect. The use of so-called calibrator oligos is a way to enhance the resolution by minimizing the technical variability between samples [8] (Fig. 3).

1.3 Instruments for HRMA

Most real-time PCR cyclers now have the option to run an extended melting program to acquire more data points. It is the accuracy of temperature control and fluorescence measurement that defines the resolution of an instrument. The ability to measure at a data

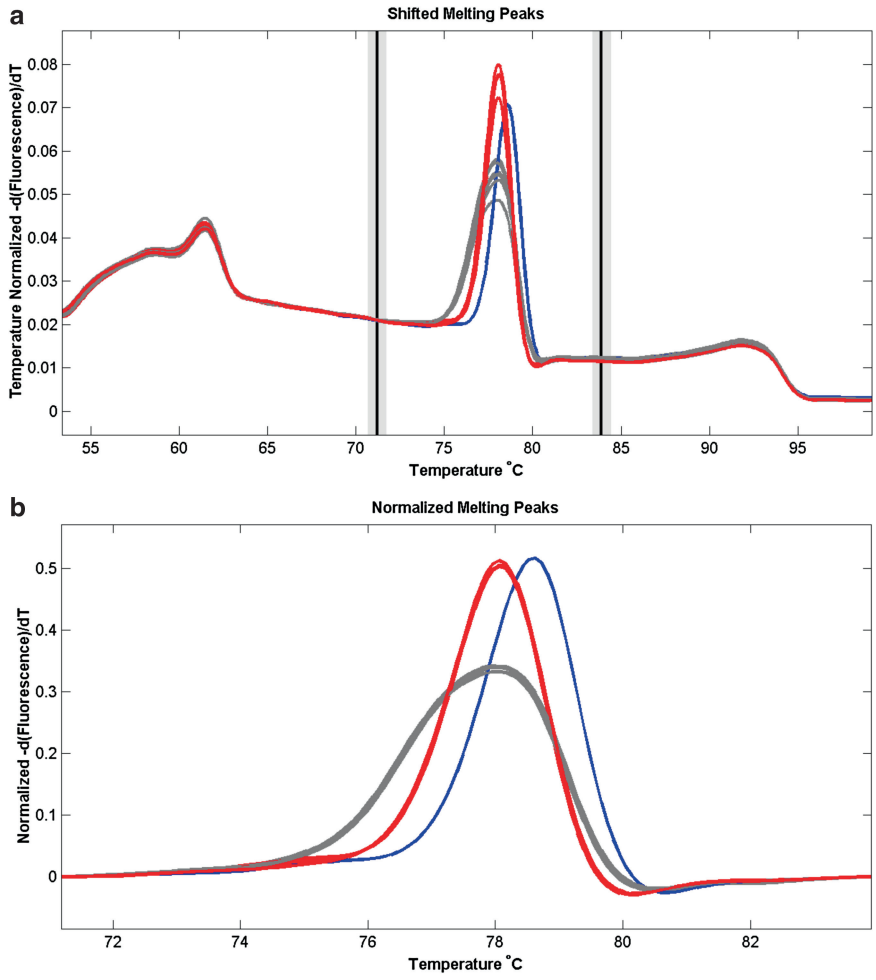

Fig. 3 Small amplicon assay, targeting a SNP and discriminating all three variants. (**a**) Shows the whole temperature range including the melting peaks from low- and high calibrator oligos from low- and high calibrator oligos (at 61 and 93 degrees respectively). (**b**) Shows the three variants. Note that the heterozygous sample (*gray*) is not resolved into two peaks. This is very common with small amplicon assays as the resolution is lower compared to unlabeled probes

density of more than 10 points/°C enhances the resolution, and is needed for HRMA [9]. Dedicated instruments for HRMA still have an advantage over general equipment [9], also because the software for those instruments is usually dedicated to HRMA and offer more analysis options. We have used the LightScanner®-96 from BioFire (formerly Idaho Technologies).

1.4 Fluorescent Dyes for HRMA

There are a few saturating DNA binding dyes available that are suitable for HRMA. We have successfully used LCGreen Plus+ (BioFire), Syto-9 (Invitrogen), and LightCycler® 480 ResoLight Dye (Roche Life Science), with a slight preference for LCGreen Plus. Others have shown Syto-9 to be comparable with LCGreen Plus [10].

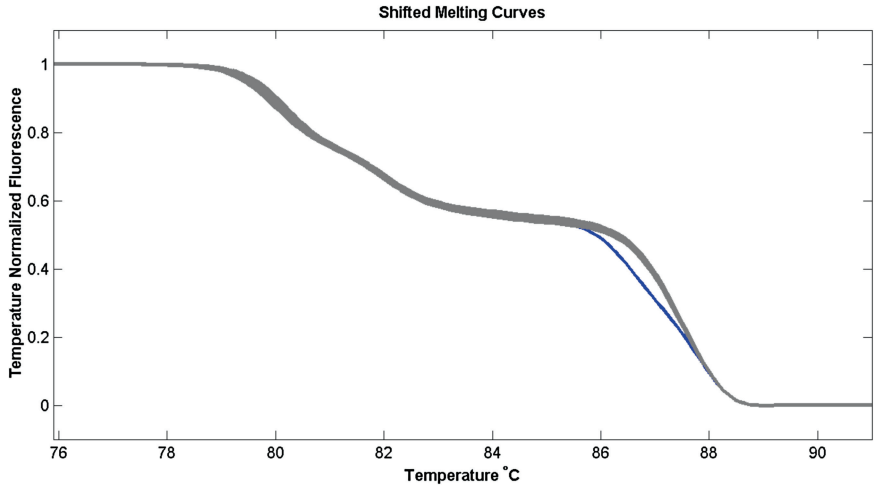

Fig. 4 Melting curve showing multiple melting domains. A variant (*blue*) is detected in the last domain

1.5 Assay Optimization

The key to success in HRMA is a well optimized PCR reaction. Any new primer design should carefully be tested with different annealing temperatures, by running a temperature gradient from, e.g. 56 °C to 68 °C. The presence of a dye such as LCGreen stabilizes DNA duplexes, and slightly raises the optimal annealing temperature. Most targets will work well at an annealing temperature of 60 °C. A well optimized amplicon gives a clean single melting peak in HRMA or band on an agarose gel. The presence of double melting domains makes it more difficult to judge the PCR conditions, as the melting curve can have more than one transitions (Fig. 4). If in any doubt, it is always useful to inspect the PCR product on a 2% agarose gel.

Additives such as 10% DMSO or 0.5 M Betaine can greatly improve the PCR conditions of amplicons with high GC%. Complete melting of a fragment may not be achieved due to high GC content. Addition of DMSO is then needed to lower the T_m. The melting behavior in a reaction is also dependent on the reaction chemistry and salt concentration. Different PCR mixes may give different results, and it is therefore important not to mix different chemistries in a single experiment. The salt concentration of the DNA sample also has an effect on the T_m. It is not recommended to compare DNA samples that were processed with different isolation methods, since differences in salt concentration will lead to variable results. It is also important to keep a similar amount of input DNA in all reactions, as big differences in DNA quantity will give less reproducible results.

The addition of a concentrated Tris/KCl solution can improve results that initially are variable [11] (Fig. 5). 1 µl of a Tris/KCl solution (1 M KCl, 0.5 M Tris–HCl pH 8) is added to the reactions post-PCR, followed by incubation of 2 min at 95 °C. After

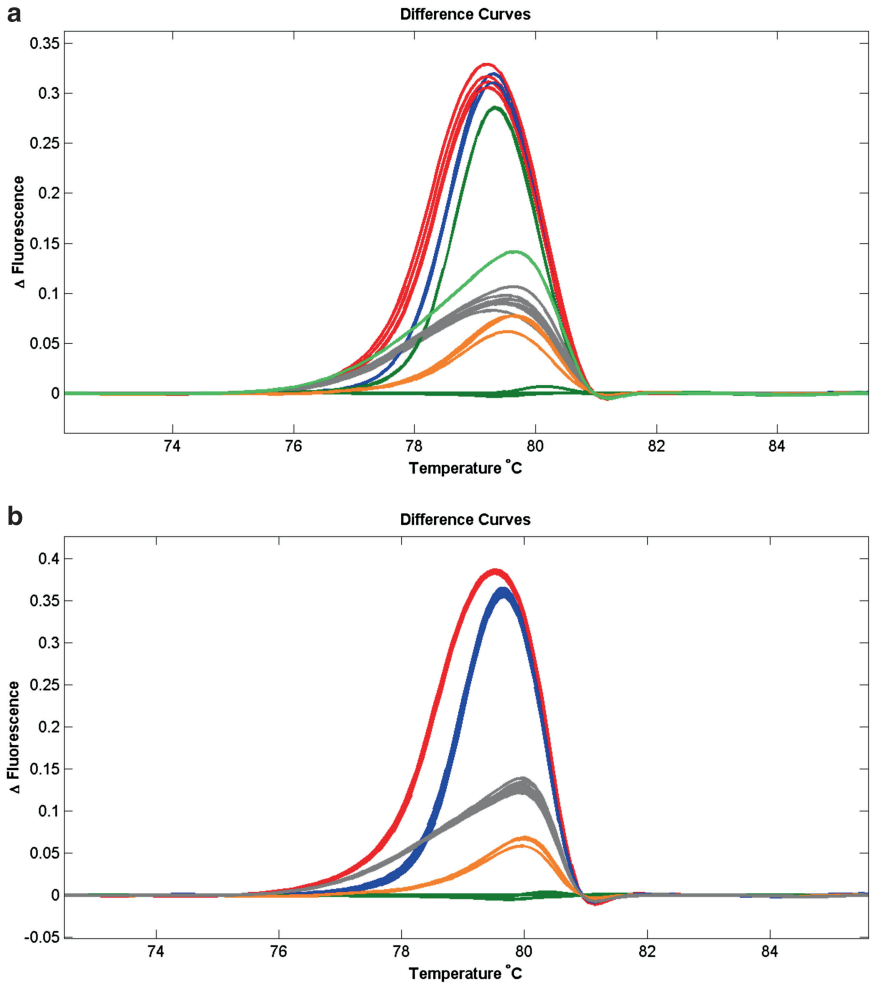

Fig. 5 Effect of the addition of a Tris/KCl solution. (**a**) Before addition of Tris/KCl. (**b**) After addition of Tris/KCl. Identical melting curves cluster much better after the addition of the solution

cooling, melting is repeated. Unfortunately, the effect can be slightly unpredictable: in some assays it will work while in other cases no improvement is seen.

2 Materials

Although several sources recommend the use of HPLC purified PCR primers, we have obtained excellent results with standard desalted oligos.

2.1 Consumables for Use with the LightScanner®-96

1. FrameStar™ 96-well skirted plates (black frame/white well, 4titude).
2. Aluminum or plastic foils.

3. Mineral oil, PCR reagent (Sigma-Aldrich).
4. FastStart Taq Polymerase (5 U/μl, Sigma) with 10× PCR reaction buffer and 20 mM $MgCl_2$ (*see* **Note 1**).
5. LCGreen Plus+ (BioFire) (*see* **Note 2**).
6. Optional: Calibrator oligos.
   Low calibrator oligo: TTAAATTATAAAATATTTATAATAT TAATTATATATATATAAATATAATA-Amine-C6
   High calibrator oligo: GCGCGGCCGGCACTGACCCGA GACTCTGAGCGGCTGCTGGAGGTGCGGAAGCGGAGG GGCGGG-Amine-C6
7. Optional: Tris/KCl solution: 1 M KCl, 0.5 M Tris–HCl pH 8.

2.2 Equipment

1. HRMA instrument, e.g. LightScanner®-96 (BioFire).
2. Thermocycler.
3. Centrifuge for spinning 96-well plates.

3 Methods

Ideally, DNA samples should be diluted to the same concentration, e.g. 10 ng/μl (*see* **Note 3**).

3.1 PCR for Scanning and Small Amplicon Analysis

1. Set up the PCR for scanning or small amplicon analysis, preparing the following mix for one reaction (10 μl reaction volume):
   - 1 μl 10× PCR-buffer 20 mM $MgCl_2$
   - 0.2 μl dNTPs (10 mM)
   - 0.3 μl F-primer (10 pmol/μl)
   - 0.3 μl R-primer (10 pmol/μl)
   - 1 μl LC-Green Plus (*see* **Note 4**)
   - 0.1 μl FastStart-Taq DNA Polymerase
   - add H_2O to 8 μl

   Optional: 0.1 μl low and/or high calibrator oligos (10 pmol/μl), for small amplicon analysis only.
2. Pipet 15 μl mineral oil in the wells of a white 96-well plate, and add 8 μl PCR-mix below the oil. Add 2 μl DNA (10 ng/μl) and seal the plate with an aluminum or plastic foil. Spin the plate briefly in a plate centrifuge.
3. Perform the following PCR program:

   10 min 95 °C

   40 cycles: 20 s 95 °C

   30 s 60 °C (*see* **Note 5**)

   40 s 72 °C

5 min 72 °C

1 min 95 °C (final denaturation before cooling to RT, stimulates heteroduplex-formation)

Cool to room temperature

3.2 PCR for Unlabeled Probe Analysis

1. Setup the PCR reaction (mix for one reaction, 10 µl reaction volume) with a 1:5 forward to reverse primer ratio.
   - 1 µl 10× PCR-buffer 20 mM MgCl2
   - 0.2 µl dNTPs (10 mM)
   - 0.1 µl F-primer (10 pmol/µl)
   - 0.5 µl R-primer (10 pmol/µl)
   - 0.5 µl probe (10 pmol/µl)
   - 1 µl LC-Green Plus
   - 0.1 µl FastStart-Taq DNA Polymerase
   - add H2O to 8 µl
2. Pipet 15 µl mineral oil in the wells of a white 96-well plate and add 8 µl PCR-mix below the oil. Add 2 µl DNA (10 ng/µl) and seal the plate with an aluminum or plastic foil. Spin the plate briefly in a plate centrifuge.
3. Perform the following PCR program:

   10 min 95 °C

   55 cycles: 20 s 95 °C

   30 s annealing temperature

   40 s 72 °C

   5 min 72 °C

   1 min 95 °C

   Cool to room temperature

3.3 Melting Acquisition and Data Analysis

After PCR, melting is performed in a machine capable of doing HRMA. In the LightScanner®-96, melting is performed at a rate of 0.1 °C/s. The temperature range at which melting is performed can vary per target and assay type. For scanning and small amplicon analysis, one can start with a broad temperature range of 60 °C–98 °C, which enables complete melting acquisition for most targets. Once the melting transition for a certain target is known, one can set a more precise temperature range to shorten the time that is needed for data collection. Unlabeled probes will dissociate earlier than amplicons and a lower starting temperature is needed, e.g. 55 °C. When using low calibrator oligos, the starting temperature can be as low as 50 °C. After data collection, melting curves are normalized by selecting a linear region before and after the melting transition (Fig. 6). Finally, temperature shifting of melting curves is done to eliminate

temperature differences between samples [12] (Fig. 7). Data analysis of melting data can be quite intuitive and one has to experiment with the parameter settings to achieve the best grouping of identical curves.

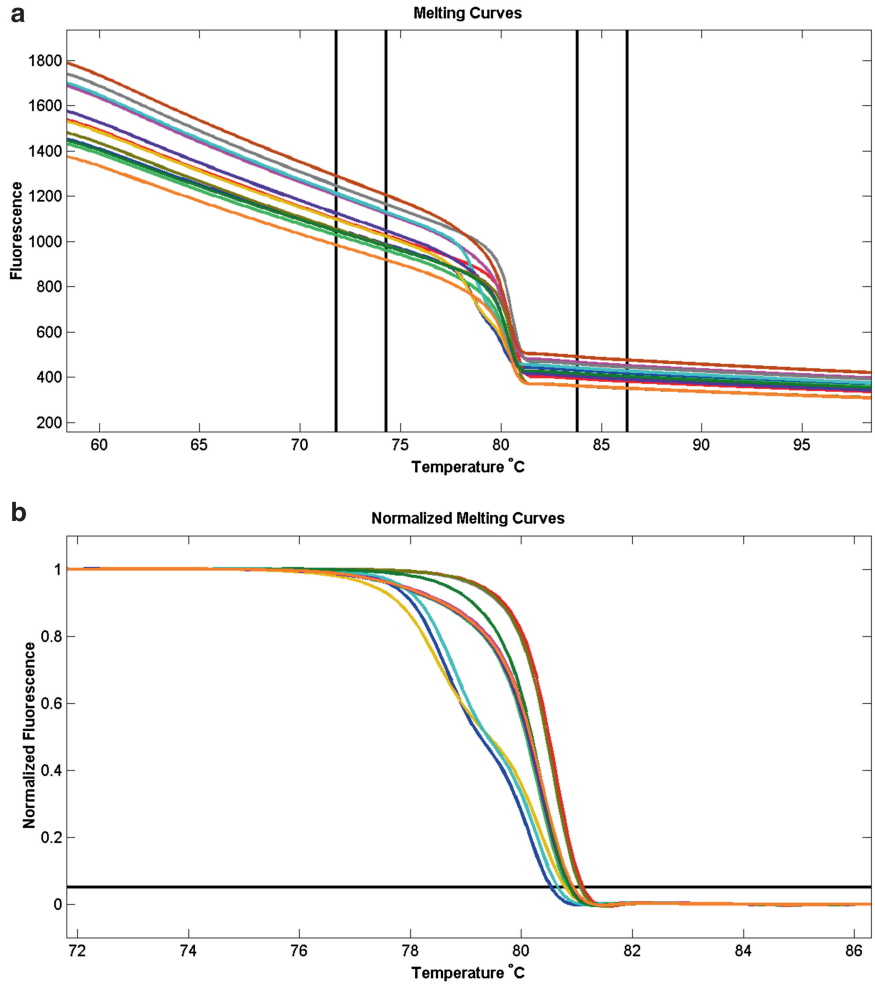

Fig. 6 (**a**) Selection of linear region before and after the melting transition. (**b**) Melting curves after normalization

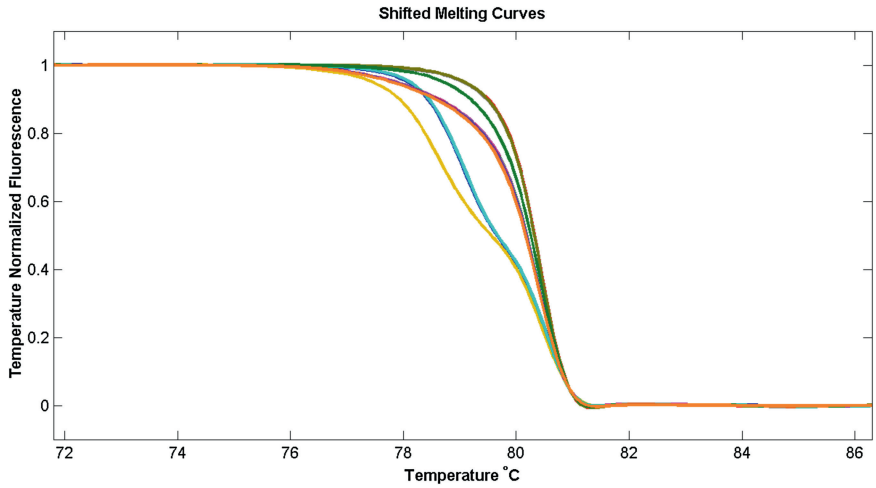

Fig. 7 Temperature shifted melting curves

4 Notes

1. The use of a hot-start Taq DNA polymerase is strongly recommended.
2. The addition of LCGreen Plus may lead to different optimal PCR conditions: re-optimization of a previously working PCR is often needed. The optimal $MgCl_2$ concentration for most targets is 2 mM.
3. When the experimental setup will allow for it, running technical duplicates is always a good idea, especially when there are differences in the amount or the quality of the DNA.
4. It is possible to add LC-Green post-PCR to an already working PCR. This is only recommended for small-scale experiments, since an extra step is added. Furthermore, adding LC-Greens will increase variation due to small differences in pipetting volumes. To add LCGreen Plus, mix 9 µl PCR product with 1 µl LCGreen Plus, incubate 3 min at 95 °C, and cool to room temperature.
5. When optimizing the PCR for many different fragments, a touch-down PCR could be considered, saving the work that is needed to optimize every fragment individually. During touch-down PCR, the annealing temperature is gradually lowered in every cycle. As an example, across the range of 40 PCR cycles one could start with 65 °C and end with 53 °C.

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