Unlabeled oligonucleotide probes modified with locked nucleic acids for improved mismatch discrimination in genotyping by melting analysis

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With a frequency of 1 in 1000 bp, single nucleotide polymorphisms (SNPs) are used to study complex inherited diseases (1,2). A universal concern in genotyping techniques is that rare variants may interfere. Melting analysis addresses this concern and is a homogeneous and simple method for genotyping (3,4). For example, the 2-probe/2-fluorophore system HybProbe® (Roche Diagnostics, Indianapolis, IN, USA) detects unexpected variants under probes by melting temperature (T_m) shifts different from that of the expected mutation (5). However, without close attention, minor shifts from the expected heterozygote T_m may be ignored, resulting in false-positive interpretations (6). Furthermore, synthesis of a labeled probe set is time-consuming and expensive. Another option is melting analysis of PCR products using double-stranded DNA (dsDNA) dyes to screen for sequence alterations. This option costs less but may be prone to error unless high-resolution techniques are used (7). Extra processing steps such as adding urea to enhance melting resolution require opening the tubes, increasing the risk of cross-contamination (8).

A recently introduced DNA dye, LCGreen®I (Idaho Technology, Salt Lake City, UT, USA), is superior to SYBR® Green I for detecting multiple products (9) and allows closed-tube genotyping with unlabeled oligonucleotide probes (10). Although high-resolution melting is not an absolute requirement for unlabeled probe genotyping, conventional real-time instruments may not distinguish between multiple variants when the variant T_m is close to the targeted mutation T_m. To further increase mismatch discrimination on the LightCycler® (Roche Diagnostics), we demonstrate the use of locked nucleic acids (LNA) (11) in unlabeled probe genotyping. LNAs incorporated into unlabeled probes increase their T_ms. As a result, mismatch destabilization is greater than conventional probes, and different mismatches are often easier to discriminate. We present a highly specific genotyping assay that detects Factor V Leiden (1691G>A) and discriminates three additional rare variants close to the mutation site using a single unlabeled (LNA-modified) probe and the LightCycler.

The assay is based on the amplification of a 151-bp PCR product using primers designed with Primer3 software (12). Unlabeled LNA-modified probes were synthesized by Integrated DNA Technologies (Coralville, IA, USA), and sequences of the probes are listed in Table 1. All probes were 31 nucleotides long with a GC content of 42%. DNA from 13 genotyped and de-identified clinical samples at the factor V Leiden locus (5 wild-types, 4 homozygous mutants, and 4 heterozygous mutants) was extracted using the MagNA Pure system (Roche Diagnostics). Three additional sequence-confirmed samples with nontargeted variants (1689G>A, 1690C>T, and 1690delC) were used for melting peak comparison. These variants were identified through routine analysis of patient samples by the LightCycler and confirmed by bidirectional sequence analysis. The 1691 position was wild-type for all three variants.

Asymmetric PCR was performed in 25-µL reactions (final volume) using PuReTaq Ready-To-Go™ PCR beads (Amersham Biosciences, Piscataway, NJ, USA) following the manufacturer's instructions. For each PCR mixture, 2 µL extracted genomic DNA (100–150 ng) were used along with 2.5 µL 10× LCGreen I for fluorescence detection. The forward primer, reverse primer, and unlabeled LNA probe concentrations were 0.05, 0.5, and 0.5 µM, respectively. PCR was performed in a GeneAmp® 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following conditions:

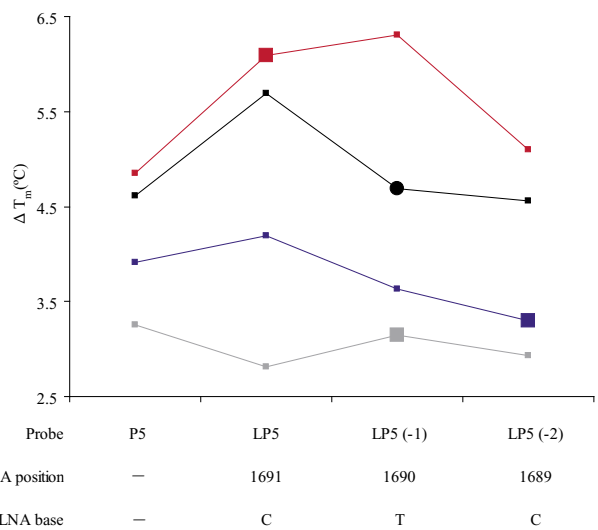

Figure 1. The effect of locked nucleic acid (LNA) position on mismatch discrimination of sequence variants. The red, blue, gray, and black lines indicate the heterozygous DNA analyzed: 1691G>A, 1689G>A, 1690C>T, and 1690delC, respectively. The probes used are indicated on the x-axis by probe name, LNA position, and LNA base. Enlarged squares indicate the mismatch positions, and the enlarged circle indicates the deletion position. ΔT_m, difference in melting temperature.

Table 1. Oligonucleotides Used in this Study

Name	Function	Sequence
Forward	PCR primer	5′-CCCATTATTTAGCCAGGAGA-3′
Reverse	PCR primer	5′-GCCTCTGGGCTAATAGGACT-3′
P5	Unlabeled probe	5′-TTCAAGGACAAAATACCTGTATTCCT**C**GCCT-3′
LP5	Unlabeled LNA probe	5′-TTCAAGGACAAAATACCTGTATTCCT**C**GCCT-3′
LP5 (-1)	Unlabeled LNA probe	5′-TTCAAGGACAAAATACCTGTATTCCT**C**GCCT-3′
LP5 (-2)	Unlabeled LNA probe	5′-TTCAAGGACAAAATACCTGTATTCC**T**C**C**GCCT-3′

Lowercase bold font indicates the position of the Leiden mutation (1691G>A). The underlined characters indicate the base replacement with locked nucleic acid (LNA). The number in parentheses in the probe name designates the number of base pairs between the LNA base and the Leiden mutation position.

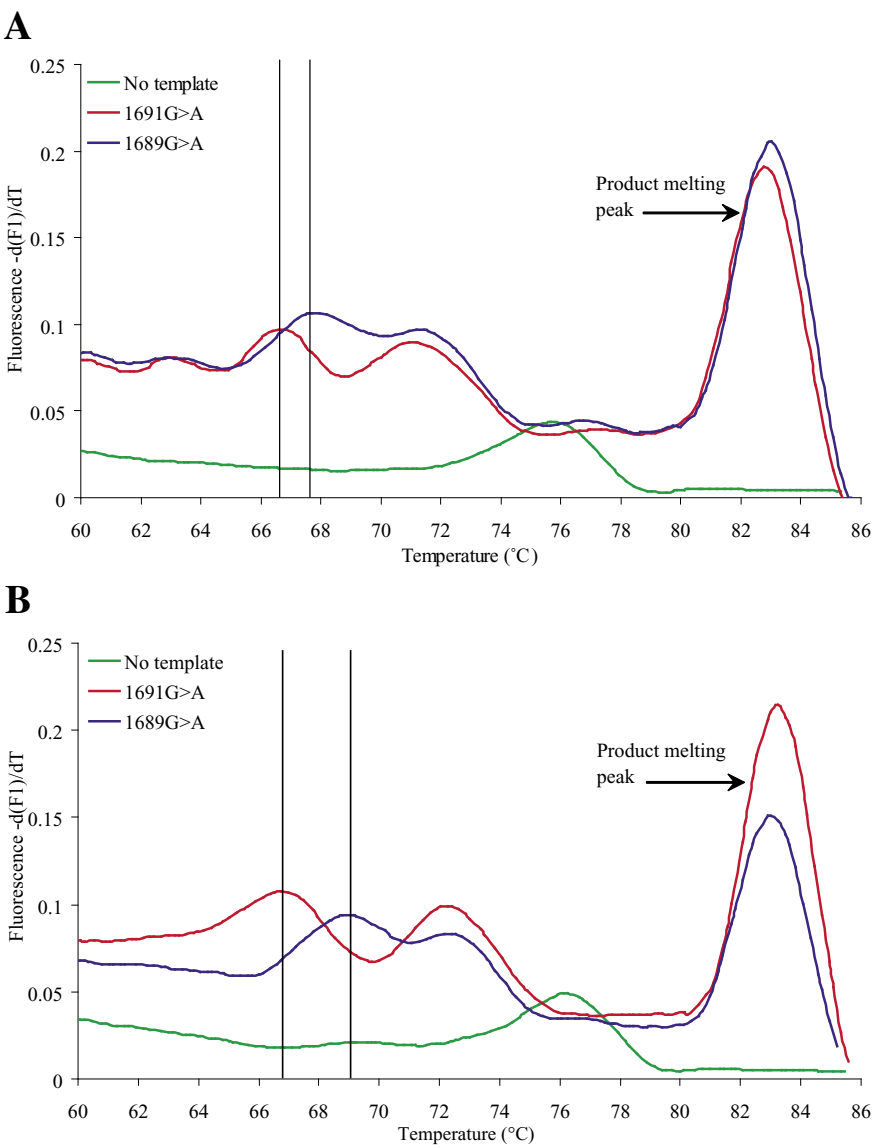

Figure 2. Derivative melting curves of heterozygous Factor V Leiden (1691G>A, red), the rare variant (1689G>A, blue), and no template controls (green). (A) An unlabeled probe without an LNA (P5) was used. (B) The probe was of the same sequence with an LNA at position 1690 [LP5 (-1)]. The probes melt at low temperatures (66°–73°C), while the products melt at higher temperatures (80°–86°C). The vertical black lines indicate the melting temperatures (T_ms) of the mismatched alleles. LNA, locked nucleic acid.

5 min at 95°C, followed by 40 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, then 72°C for 5 min (final extension), 95°C for 3 min (final denaturation), and then cooled to 4°C. Ten microliters molecular biology-grade mineral oil (Sigma, St. Louis, MO, USA) were also added to each PCR mixture to prevent evaporation during cycling and melting. After amplification, products were transferred to glass capillaries and subjected to melting analysis in a LightCycler, with data acquisition from 55° to 90°C and a thermal transition rate of 0.1°C/s. Data analysis was performed using LightCycler software to display derivative peaks. A 96-well thermal cycler was used for PCR amplification (instead of the LightCycler) in anticipation of ultimately melting samples in 96- or 384-well plates on a LightScanner™ (Idaho Technology) instead of glass capillaries.

The effect of LNA position on mismatch discrimination of Factor V Leiden (1691G>A) is shown in Table 2. Without LNA base incorporation, the ΔT_m (difference in melting temperature) between the matched and mismatched peaks of heterozygotes was 4.85°C. When an LNA was incorporated at the position of the targeted mutation, the ΔT_m increased to 6.09°C, suggesting a better mismatch discrimination primarily from LNA stabilization of the perfectly matched hybrid. When the LNA base was moved one (-1, LNA base on 1690 position) or two (-2, LNA base on 1689 position) bases away from the mutation position, the ΔT_m s were 6.31° and 5.10°C, respectively. The (-1) position resulted in the greatest mismatch discrimination.

To test if the position adjacent to the sequence variant is always the best base for LNA modification, three additional nontargeted rare genotypes were tested (1689G>A, 1690C>T, and 1690delC) (Figure 1). The best mismatch discrimination (the highest ΔT_m) for genotype 1689G>A occurred with the LNA base two bases away (position 1691). For genotype 1690C>T, the presence and position of the LNA in the probe had little effect on ΔT_m. For genotype 1690delC, an adjacent LNA base on one side (position 1689) of the mutation had no effect, while an

Table 2. Effect of LNA Incorporation and Position on the T_m of Unlabeled Probes Used for Genotyping Factor V Leiden (1691G>A)

Probe	Mismatched Peak T_m (°C)	Matched Peak T_m (°C)	ΔT_m (°C)
P5	66.47	71.32	4.85
LP5	66.33	72.42	6.09
LP5 (-1)	66.11	72.42	6.31
LP5 (-2)	67.57	72.67	5.10

LNA, locked nucleic acid; ΔT_m, difference in melting temperature.

LNA base on the other side (position 1691) increased ΔT_m about 1°C. In addition to LNA position relative to the mutation, the SNP type and LNA base may also affect probe T_m in complex ways, making predictions difficult.

The ability to distinguish different sequence variants depends on their difference in T_m. Inspection of Figure 1 shows that probe LP5 (-1) has the best spacing between genotypes. Figure 2 shows derivative melting curves of two genotypes comparing the LP5 (-1) probe to the probe without the LNA (P5). Better discrimination between genotypes was obtained with the LNA probe. All four genotypes were separated by at least 0.5°C, allowing genotyping of all four variants on the LightCycler without high-resolution analysis. In addition to analysis of the unlabeled probe melting transition, the product melting transition can be used for genotyping, either as secondary confirmation of the unlabeled probe genotype (13), or as the sole means of genotyping (14). However, a high-resolution instrument such as the HR-1 (Idaho Technology) is required to genotype by product melting patterns.

LNA bases reportedly work best with oligonucleotides less than 10 bases (15). However, Zhou et al. (10) indicate that longer unlabeled probes are best for genotyping. Probes of 20–30 bp, small products, and asymmetric PCR (with 40–50 PCR cycles) enhance fluorescence intensity when genotyping with unlabeled probes (10). Incorporating LNAs increases mismatch discrimination so that conventional real-time instruments can be used with greater confidence. When the temperature difference between wild-type and mutant alleles is increased, it is more likely that different heterozygous variants can be distinguished. Using LNA unlabeled probes, four different heterozygous variants near the factor V Leiden locus (1691G>A, 1689G>A, 1690C>T, and 1690delC) were easily identified. This simple genotyping method includes both the advantages of unlabeled oligonucleotide probes (low expense, simple design) and LNA-modified nucleic acids (better allele discrimination) (11,15–17). The approach uses only one probe and requires no fluorescent labeling, decreasing the time and cost for oligonucleotide synthesis.

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COMPETING INTERESTS STATEMENT

Aspects of high-resolution melting analysis are licensed from the University of Utah to Idaho Technology. C.T.W. holds equity interest in Idaho Technology. L.-S.C., C.M., and E.L. declare no competing interests.

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