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 (Tm) shifts different from that of the expected mutation (5). However, without close attention, minor shifts from the expected heterozygote Tm 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® (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 Tm is close to the targeted mutation Tm. 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 Tm's. 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:

- PCR temperature ramp: 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min
- Final extension: 72°C for 7 min
- Analysis: Melting curve analysis at 95°C

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. ΔTm difference in melting temperature.
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°C 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°C 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
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REFERENCES


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