Title: **SNP Genotyping by Unlabeled Probe Melting Analysis**

Running Title: **Unlabeled Probes**

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i. Summary

Fluorescent nucleic acid detection in PCR generally uses oligonucleotide probes labeled with dyes. However, unlabeled oligonucleotides in the presence of saturating DNA dyes can also serve as hybridization probes. The DNA dye, LCGreen Plus, and a 3'-blocked unlabeled probe are added before amplification, and asymmetric PCR is performed at a 1:5 to 1:10 primer ratio. After PCR is complete, fluorescent melting curves reveal both probe melting at low temperature and amplicon melting at high temperature. After background removal, the melting temperature(s) of the probe/target duplex specific to the allele(s) amplified are revealed. Probes between 20 and 40 bp with Tms between 50 and 85 °C are effective. The method requires only three standard oligonucleotides and endpoint fluorescence melting. No real-time PCR or allele-specific amplification is required. Unlabeled probes are inexpensive, provide the sequence specificity of probes, and allow simultaneous identification of multiple alleles by melting analysis.

ii. Key words: unlabeled probes, genotyping, LCGreen Plus, asymmetric PCR, melting analysis

1. Introduction

Dye methods remain very popular in real-time PCR, even without the specificity of an internal probe. The reason is simple. Fluorescent probes with covalently attached labels are expensive irrespective of the specific design. In addition to the expense, they require more time to obtain from commercial suppliers, quality control is problematic,

and a new probe is needed for each target of interest. In contrast, a DNA dye can be used for any target. Furthermore, product melting analysis can identify the amplified product with an accuracy that depends on the resolution of the melting instrument (Herrmann et al. 2006). Even so, probes remain preferred by many, especially in clinical diagnostic applications. A method with the specificity of a probe and the cost and simplicity of a closed-tube dye analysis would be desirable.

The dye, SYBR® Green I, was first used in real-time PCR in 1997 (Ririe et al. 1997, Wittwer et al. 1997). SYBR Green I is commonly used to detect duplex PCR products. In addition, SYBR Green I can successfully detect probe/product melting if single stranded product is isolated and immobilized (Jobs et al. 2003, Prince et al. 2001). Although processing is required, genotyping by melting is possible without labeled probes. However, SYBR Green I melting analysis is limited in closed-tube systems when multiple duplexes are present in solution. Higher Tm products are preferentially detected and heteroduplexes are not observed at dye concentrations compatible with PCR (Wittwer et al. 2003). Indeed, genotyping in solution with unlabeled probes after asymmetric PCR was not successful when SYBR Green I was used (Zhou et al. 2004a). At PCR compatible concentrations, SYBR Green I does not saturate all DNA duplexes present and the dye appears to redistribute to higher Tm duplexes during melting.

The LCGreen® family of dyes detects all duplexes present in solution because saturating concentrations can be used that do not inhibit PCR (Wittwer et al. 2004). Unlabeled probes are included in PCR that are not extended by polymerase because they are 3'-blocked. After asymmetric PCR, the probes anneal to single-stranded product. Melting curves show regions of both probe/product and product/product melting (Zhou et

al. 2005). Different alleles result in different probe/product melting transitions based on the stability of the mismatches present (Zhou et al. 2004a). It is easiest to see the transitions by plotting the negative derivative (-dF/dT) of fluorescence (F) vs temperature (T). Figure 1 diagrams the method. Often, background fluorescence is high, resulting in an elevated background, especially at low temperatures.

Background fluorescence can be optimally subtracted by fitting a decreasing exponential to the slope of the curve in regions where no melting occurs. Although exponentials are usually fit to values rather than slopes, both approaches require two equations with two unknowns. Since the contribution of background to total fluorescence is not known, fluorescence values cannot be used. However, in regions where no melting occurs, the slope of the curve is entirely attributable to background because the slope contribution from melting is zero. This exponential approach is superior to linear baseline normalization (Wittwer and Kusukawa 2004) in removing background fluorescence from unlabeled probe and combined unlabeled probe/amplicon melting curves that cannot be normalized using baselines. It also provides better background removal when multiple small amplicons are analyzed ((Liew et al. 2006, Liew et al. 2004).

The following procedure is for unlabeled probe genotyping of the common clinical target, factor V Leiden, a risk factor for coagulation. Any SNP or small deletion/insertion can be genotyped by similar means.

2. Materials

2.1 DNA Isolation (see **Note 1**)

1. Sample: EDTA/ACD/sodium citrate/sodium heparin anti-coagulated human blood
2. QIAamp DNA Blood Mini Kit (Qiagen).
3. Ethanol (96 – 100%)

2.2 Polymerase Chain Reaction

1. 10X thermostable DNA Polymerase (*see* **Note 2**), 0.4 U/µl, diluted from concentrated stock in Taq dilution buffer (2.5 mg/ml BSA, 10 mM Tris, pH 8.3, Idaho Technology). For example, Taq (Roche Applied Science) or KlenTaq1 (AB Peptides).
2. 10X PCR buffer, 500 mM Tris, pH 8.3, 2.5 mg/ml BSA, 30 mM $MgCl_2$ (Idaho Technology).
3. 10X dNTPs: 2 mM each of dATP, dCTP, dGTP and dTTP (Idaho Technology) (*see* **Note 3**).
4. 10X LCGreen Plus (Idaho Technology) (*see* **Note 4**)
5. 10X primers/probe (*see* **Notes 5/6**):

   Factor V forward primer (5 uM): CTGAAAGGTTACTTCAAGGAC

   Factor V reverse primer (1 uM): GACATCGCCTCTGGG

   Factor V probe (4 uM): TGGACAGGC**G**AGGAATACAGGTT-P

2.3 Instrumentation (*see* **Note 7**)

1. GeneAmp® PCR System 9700 (Applied Biosystems)
2. LightCycler® (Roche)

3. LightScanner™ (Idaho Technology)
4. HR-1™ Instrument (Idaho Technology)

3. Methods

3.1 DNA Isolation

1. Whole blood obtained in EDTA, ACD, sodium citrate, or sodium heparin tubes is processed according to the QIAamp DNA Blood Mini Kit Handbook.
2. Measure the absorbance at 260 nm and adjust to an absorbance of 1.0 (50 ng/ul).

3.2 PCR Preparation (*see* **Note 8**)

1. Mix one part of each of the following 10X solutions with four parts water: DNA polymerase, PCR buffer, dNTPs, LCGreen Plus, and primer/probe mix.
2. Aliquot 9 parts of the above master mix into each well or capillary.
3. Add 1 part of DNA to each well or capillary.

3.3 PCR Amplification in 96/384 plates

1. Overlay each well with 10 ul (384-well) or 15 ul (96-well) of mineral oil.
2. Briefly spin the plate in a centrifuge.
3. Amplify with an initial denaturation of 94 °C for 10 s followed by 50 cycles of 94 °C for 5 s, 57 °C for 2 s, and 72 °C for 2 s.
4. After amplification, heat to 94 °C for 1 s then cool to 10 °C before melting.

3.4 PCR Amplification in capillaries

1. Spin all samples down into the capillaries on a centrifuge.
2. Amplify on a LightCycler with an initial denaturation of 94 °C for 10 s followed by 50 cycles of 94 °C for 0 s, 55 °C for 0 s, and 72 °C for 2 s.
3. After amplification, heat to 94 °C for 1 s then rapidly cool at 20 °C/s to 40 °C before melting.

3.5 Melting Acquisition on the LightScanner

1. Transfer the plate from the thermocycler to the LightScanner.
2. Heat the plate from 55 °C to 88 °C at 0.1 °C/s, giving ~25 points/°C.

3.6 Melting Acquisition with HR1

1. Transfer each capillary to the HR-1 high-resolution melting instrument.
2. Perform melting from 55 °C to 88 °C with a slope of 0.3 °C/s, giving 65 points/°C.

3.7 Melting Analysis

Software on most instruments allows visualization of probe and product melting transitions as derivative peaks, usually by Salvitsky-Golay polymonial estimation of the slope at each point (Wittwer and Kusukawa 2004). Analysis of a 384-well run for factor V Leiden is shown in Fig. 2. Part A shows the data without background subtraction, both as the original melting curve (top) and its derivative (bottom). Part

B shows the data after exponential background subtraction (*see* **Note 9**), both as a normalized melting curve (top) and a derivative plot (bottom).

4. Notes

1. Although a common commercial DNA preparation kit is referred to here, any DNA purification procedure can be used.
2. Various Taq polymerases can be used, including Taq DNA Polymerase (Roche Applied Science), KlenTaq1™ (AB Peptides) with TaqStart™ antibody (Clontech), and FastStart Taq DNA polymerase (Roche). A chemically modified hot start Taq polymerase or the addition of TaqStart antibody is not necessary, but makes the PCR more robust. However, whether the polymerase has 5'-exonuclease activity will impact the design of the probes. If a 5'-exo-negative polymerase is used, the probe can block enzyme extension if it is bound to the template during enzyme extension. Therefore, the probe Tm should be lower than the PCR extension temperature (<70°C). If a 5'-exo-positive polymerase is used, the probe Tm can be as high as 85°C.
3. A dNTP mix that includes dUTP may also be used and will shift the probe Tm to lower temperatures. If the total dNTP concentration changes, adjust the $MgCl_2$ concentration accordingly. Uracil-N-glycosylase may also be included (Liew et al. 2004) although it is usually not considered necessary in closed-tube systems (Wang et al. 2005).
4. Saturating dyes, LCGreen® I or LCGreen® Plus (Idaho Technology) can be used for unlabeled probe genotyping. These dyes differ from the commonly used

SYBR® Green I in their ability to be used at high concentrations, allowing all available double-stranded binding sites to be saturated while not inhibiting PCR amplification. The excitation maximum for the LCGreen dyes is 450 nm with emission at 470 nm, allowing use on the SYBR Green I channel of most real-time instruments. However, the LCGreen dyes are not excited by argon-ion laser-based instruments (Herrmann et al. 2006). The use of LCGreen dyes increases the melting temperature of DNA probes by about 1-3 °C, and adjustment of cycling parameters may be required. LCGreen Plus has higher fluorescence intensity than LCGreen I and is best used on plate-based instruments.

5. Primers are prepared with standard desalting. Unlabeled probe signals are stronger with shorter PCR products. Amplicons less than 100 bp are optimal, while 200 bp amplicons still give strong signals and most probes within 400 bp amplicons can still be used. Asymmetric PCR (1:5 to 1:10) produces both double-stranded amplicon and the appropriate single-stranded DNA for probe binding (Figure 1).
6. Probes are blocked on the 3' end to prevent extension. The most common blocker is a phosphate. Incomplete phosphate blocking (from either incomplete synthesis or hydrolysis on storage) produces longer probes that appear as extra peaks on derivative plots between the expected probe and product melting temperatures. When this occurs, a C3 blocker may be more effective in preventing extension (Cradic et al. 2004). The length of an unlabeled probe is usually 20 to 40 bases depending on the GC content and the desired Tm (Zhou et al. 2004a). Longer probes give stronger signals. Tms between 50 and 85 °C have been successfully

used. Probe Tm can be lowered by using dUTP instead of dTTP during probe systhesis (Zhou et al. 2004b), or increased by substituting locked nucleic acids into the probe (Chou et al. 2005). As with any probe based system, mismatches destabilize the probe and lower the Tm. Irrelevant polymorphisms can be masked by incorporating mismatches, deletions, or universal bases into the probes (Margraf et al. 2006).

7. Unlabeled probes can be analyzed on any fluorescent melting instrument compatible with LCGreen dyes. Different alleles are best discriminated on instruments specifically designed for high-resolution melting analysis (HR-1 and the LightScanner). The HR-1 instrument performs melting analysis on samples contained in capillaries that were previously amplified on a LightCycler. The HR-1 processes one sample at a time with a throughput of ~ 40 samples per hour. The LightScanner analyzes 96 or 384 well plates that have been amplified in any 96 or 384 well thermocycler. The time for analysis in the LightScanner is 5-15 minutes for a full plate, depending on ramp times and temperature ranges. An extensive comparison of 9 instruments capable of melting analysis has been recently reported (Herrmann et al. 2006). In addition to the HR-1 instrument and the LightScanner, this study evaluated the melting capabilities of the ABI PRISM® 7000, ABI PRISM® 7900HT, BioRad iCycler, Cepheid SmartCycler®, Corbett Rotor-Gene™ 3000, and the Roche LightCycler 1.2 and 2.0. All but the 7900HT were compatible with unlabeled probe analysis with LCGreen Plus. Roche has recently introduced the LightCycler 480 System, which is a real time 96/384-well

PCR instrument that should also be useful for melting analysis of unlabeled probes.

8. The usual total reaction volume is 10 ul. However, reactions can be scaled down to 5 ul if desired in either capillaries or plates.

9. Exponential background subtraction is not currently incorporated into any commercial software. However, the method is straightforward and is easily implemented as follows: Fit the slope of the collective (signal plus background) curve, $F(T) = M(T) + B(T)$ at two temperatures, T_L and T_R, below and above any melting transition temperatures, so that the slope $M'(T_L)=M'(T_R)=0$ and therefore $F'(T_L) = B'(T_L)$ and $F'(T_R) = B'(T_R)$. Fit an exponential model for $B(T) = Ce^{a(T-T_L)}$, shifted to T_L for numerical stability to these two values: $B'(T) = aCe^{a(T-T_L)}$ at $T = T_R, T_L$. At $T = T_L$, this gives $aC = B'(T_L)$ and at $T=T_R$ this gives $aCe^{a(T_R-T_L)} = B'(T_R)$. Since the two values on the right hand sides have been measured, we may use them to obtain the model parameters a and C as follows. Dividing the second equation by the first gives $e^{a(T_R-T_L)} = B'(T_R)/B'(T_L)$ so that $a = \ln(B'(T_R)/B'(T_L))/(T_R-T_L)$ and the first equation gives $C = B'(T_L)/a$. Finally, obtain the signal with the background removed by subtraction: $M(T) = F(T) - Ce^{a(T-T_L)}$ with the parameters C and a determined as above. The signal M(T) may optionally be normalized to the range $0 - 100$ by applying the linear shift and rescaling $M(T) = 100(M(T) - m)/(M - m)$ where $m = \min\{M(T)\}$ and $M = \max\{M(T)\}$ on the interval of interest.

Figure Legends

Fig. 1. Genotyping by unlabeled probe melting analysis. (A) Asymmetric PCR in the presence of LCGreen dye and an unlabeled probe produces both probe/product and product/product duplexes. (B) Melting analysis reveals both low temperature (probe/product) and high temperature (product/product) melting transitions (Zhou et al. 2005). Complete SNP genotyping is possible by analysis of either region. However, probe genotyping does not require high-resolution instrumentation or analysis techniques.

Fig. 2. Unlabeled probe genotyping of factor V Leiden in 384-well format on the LightScanner. Data are displayed as melting curves (top) and derivative plots (bottom) either without (A) or with (B) exponential background subtraction. It is much easier to cluster the curves by genotype after exponential background subtraction.

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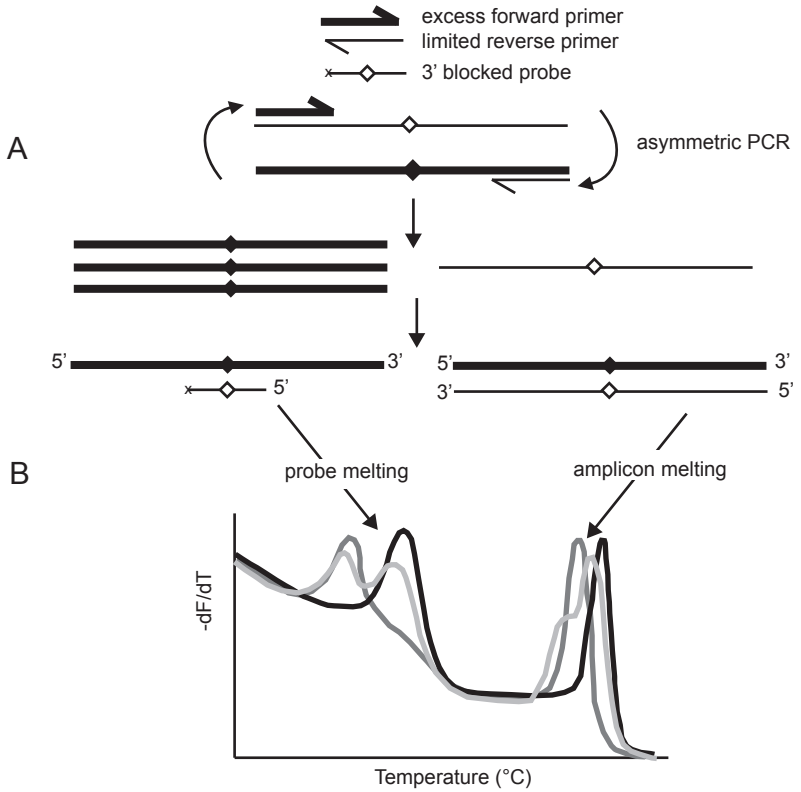
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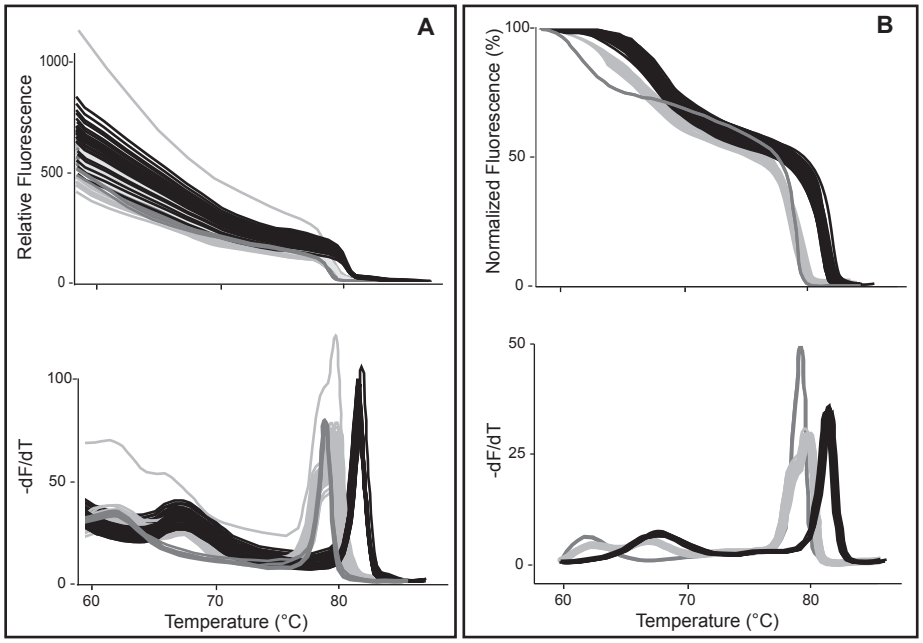
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