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Snapback Primer Genotyping with Saturating DNA Dye and Melting Analysis

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BACKGROUND: DNA hairpins have been used in molecular analysis of PCR products as self-probing amplicons. Either physical separation or fluorescent oligonucleotides with covalent modifications were previously necessary.

METHODS: We performed asymmetric PCR for 40–45 cycles in the presence of the saturating DNA dye, LCGreen Plus, with 1 primer including a 5′ tail complementary to its extension product, but without any special covalent modifications. Samples were amplified either on a carousel LightCycler for speed or on a 96/384 block cycler for throughput. In addition to full-length amplicon duplexes, single-stranded hairpins were formed by the primer tail "snapping back" and hybridizing to its extension product. High-resolution melting was performed on a HR-1 (for capillaries) or a LightScanner (for plates).

RESULTS: PCR products amplified with a snapback primer showed both hairpin melting at lower temperature and full-length amplicon melting at higher temperature. The hairpin melting temperature was linearly related to the stem length (6–28 bp) and inversely related to the log of the loop size (17–135 bases). We easily genotyped heterozygous and homozygous variants within the stem, and 100 blinded clinical samples previously typed for *F5* 1691G>A (Leiden) were completely concordant by snapback genotyping. We distinguished 7 genotypes in 2 regions of *CFTR* exon 10 with symmetric PCR using 2 snapback primers followed by product dilution to favor intramolecular hybridization.

CONCLUSIONS: Snapback primer genotyping with saturating dyes provides the specificity of a probe with only 2 primers that are free of special covalent labels in a closed-tube system.

DNA hairpins are useful tools in molecular diagnostics, including stem-loop probes *(1)* and self-probing amplicons *(2, 3)*. Stem-loop probes, commonly known as "molecular beacons," are covalently modified oligonucleotides with a fluorophore on one end and a quencher on the other. Self-probing amplicons have been used in snapback single-stranded conformation polymorphism (SSCP)[4] *(2)* and Scorpion primers *(3)*. Snapback SSCP was developed to artificially introduce secondary structure into PCR products for SSCP analysis. Dependent on the amplicon sequence, a complementary 8- to 11-bp primer tail snaps back on its extension product, forming single-stranded hairpins. Although the primers in snapback SSCP are not covalently modified, electrophoresis is necessary to separate the hairpins.

Scorpion primers have a complex 5′ extension that includes a probe element, a pair of self-complementary stem sequences, a fluorophore, a quencher, and a blocking monomer to prevent copying the 5′ extension. Intramolecular scorpions have some advantages over intermolecular beacons, because hybridization is rapid and not rate limiting, even with the fastest PCR protocols *(4)*. The hairpin is stabilized by the intramolecular reaction, increasing its melting temperature (Tm) by about 5–15 °C, so that shorter regions can be analyzed when the sequence variation is high. Furthermore, only 2 oligonucleotides are necessary, because 1 serves as both primer and probe. Both molecular beacons and scorpion primers are ingenious methods that can be used for genotyping and real-time PCR, but complexity in design, synthesis, and purification limits their use.

Instead of covalent labels, DNA dyes that fluoresce in the presence of double-stranded DNA can be used for real-time PCR and closed-tube genotyping. SYBR Green I is commonly used *(5, 6)*, and specificity may be increased by melting analysis *(7)*. The introduction

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[4] Nonstandard abbreviations: SSCP, single-stranded conformation polymorphism; Tm, melting temperature.

of high-resolution melting techniques now makes analysis with dyes even more attractive *(8)*. New saturating dyes have been developed *(9, 10)* that reliably detect heteroduplexes in PCR products *(11)*, and high-resolution melting instruments have appeared *(12–14)*. Probe-specific genotyping by melting is possible with LCGreen Plus using unlabeled probes that are modified only by blocking their 3′ end to prevent extension *(15–20)*. By incorporating the unlabeled probe into a snapback primer, the advantages of self-probing amplicons are possible without the complexity and cost of special covalent modifications.

Materials and Methods

DNA SAMPLES

PCR templates included engineered plasmids of M13 sequence *(21)* and human genomic DNA. Otherwise identical plasmids with an A, C, G, or T at 1 position were kindly provided by Lonza Rockland, Inc. We determined the concentration of each plasmid by absorbance at 260 nm (A_{260}), assuming that an A_{260} of 1.0 is 50 mg/L. We formed single-base "heterozygotes" by mixing equal molar amounts of 2 different plasmids.

We obtained DNA extracts from 100 human blood samples submitted to Associated Regional and University Pathologists for *F5* (coagulation factor V)[5] genotyping on a MagNA Pure LC (Roche). The DNA concentrations were not quantified, but ranged from 20–40 mg/L *(22)*. The samples were genotyped for *F5* 1691G>A *(22)* and deidentified according to a global ARUP protocol (IRB 7275). We obtained samples with known *CFTR* (cystic fibrosis transmembrane conductance regulator) variants from the Coriell Institute for Medical Research. Primers were synthesized by the University of Utah core synthesis facility using Trityl-ON column purification for snapback primers. Sequences of the PCR primers and lengths of the PCR product, loop, and duplex of the hairpin are listed in Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue10.

PCR AND MELTING CURVE ACQUISITION

We performed PCR of plasmid templates in 10-μL reaction volumes containing 50 mmol/L Tris (pH 8.3), 500 mg/L bovine serum albumin, 3 mmol/L $MgCl_2$, 200 μmol/L of each deoxynucleotide triphosphate, 0.4 units KlenTaq polymerase (AB Peptides), 88 ng TaqStart antibody (Clontech), 0.5× LCGreen Plus (Idaho Technology), 0.5 μmol/L of the snapback primer, 0.05 μmol/L of the limiting primer, and 10^6 copies of plasmid. PCR was performed in a LightCycler (Roche) for 45 cycles with denaturation at 95 °C (0 s hold), annealing at 50 °C (0 s hold), a 2 °C/s ramp to the extension temperature of 72 °C, and an 8 s hold at 72 °C. After PCR, the capillary samples were denatured at 94 °C (0 s hold) and cooled to 40 °C. Transition rates between temperatures were programmed at 20 °C/s unless otherwise stated. The samples were removed from the LightCycler and melted at 0.3 °C/s in the high-resolution melting instrument HR-1 (Idaho Technology).

We performed PCR from human genomic DNA as above with the following exceptions. Microtiter plates were used for PCR at template concentrations of 2–4 mg/L (*F5*) and 5 mg/L (*CFTR*). *CFTR* amplification was symmetric with each primer at 0.5 μmol/L. Reaction volumes of 10 μL (*F5* in 384-well plates) or 2 μL (*CFTR* in 96-well plates) were overlaid with 10–15 μL mineral oil (Sigma), centrifuged at 1500*g* for 3–5 min, and subjected to PCR in a PTC-200 thermal cycler (Bio-Rad). An initial denaturation was performed at 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s, 58 °C for 15 s, and 72 °C for 20 s (*F5*) or 35 cycles of 95 °C for 15 s, 55 °C for 10 s, and 72 °C for 15 s (*CFTR*). After PCR, the *CFTR* samples were diluted with water (18 μL in each well for a 10× dilution), centrifuged, heated to 95 °C in a LightScanner, removed from the instrument, and cooled to <40 °C in room air. Before melting, all plates were centrifuged, followed by fluorescence acquisition during heating at 0.15 °C/s on a 96- or 384-well LightScanner, as appropriate.

MELTING CURVE ANALYSIS

Melting curves were analyzed by methods detailed elsewhere *(9, 16, 23)*, with either custom software or commercial LightScanner software. Briefly, melting curves were normalized between 0% and 100%, exponential background was subtracted *(24)*, and melting curves were displayed as negative first-derivative plots of fluorescence with respect to temperature.

Results

A schematic of snapback primer genotyping is shown in Fig. 1. The snapback primer includes a 5′ tail that is complementary to its own extension product. After asymmetric PCR, both intramolecular snapback hairpins and intermolecular duplexes of full-length amplicons are formed. In the presence of a saturating fluorescent DNA dye, the melting of both snapback and amplicon duplexes are observed as peaks on negative first-derivative plots of fluorescence with respect to

[5] Human genes: *F5*, coagulation factor V (proaccelerin, labile factor); *CFTR*, cystic fibrosis transmembrane conductance regulator (ATP-binding cassette, subfamily C member 7).

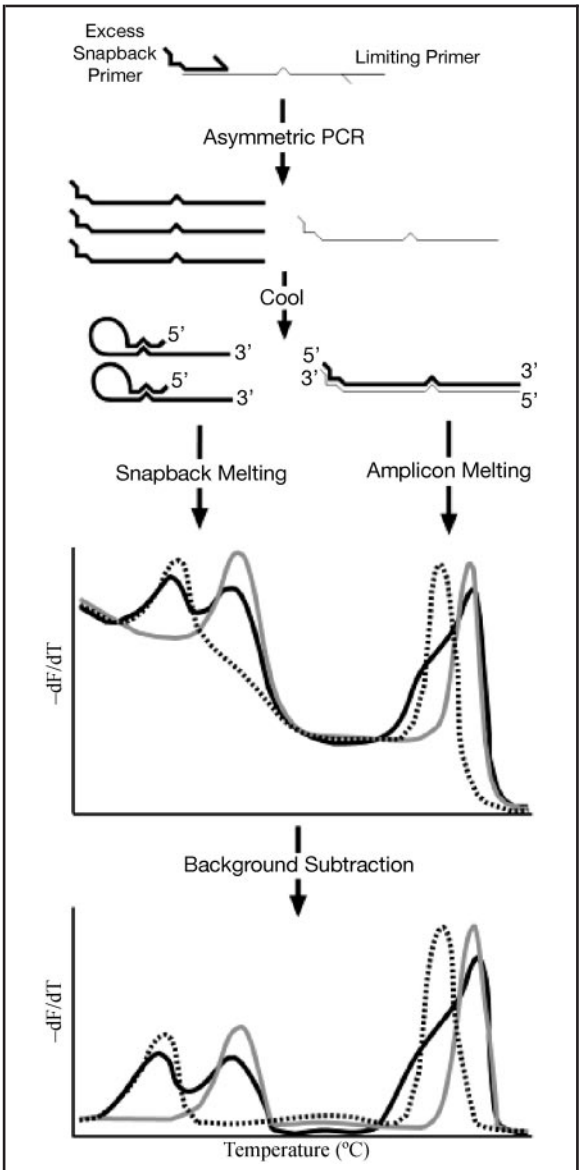

Fig. 1. Snapback primer genotyping with a saturating DNA dye.

Snapback primers are standard oligonucleotides that include as a probe element a 5′ tail that is complementary to the extension product of the primer. Asymmetric PCR is performed with an excess of the snapback primer. On cooling, both full-length amplicons and intramolecular hairpins of the excess snapback strand are formed. Melting reveals both low-temperature (snapback hairpin) and high-temperature (full-length amplicon) transitions. Exponential background subtraction removes nonspecific fluorescence from unincorporated primers. Melting of the probe element provides targeted genotyping, whereas high-resolution analysis of amplicon melting optionally detects variants anywhere within the amplicon.

temperature. Sequence variation within the hairpin stem alters the Tm of the snapback duplex, allowing genotyping by melting analysis, similar to unlabeled probe genotyping *(15)*. Variation anywhere between the primers will also alter full-length amplicon melting and can be used for variant scanning *(11, 25)*. The relative amounts of hairpin and amplicon duplex can be adjusted by the concentration of the limiting primer and the number of cycles. A 2-bp mismatch at the 5′ end of the snapback primer may also be included to prevent 3′-end extension of the minor hairpin that may form from the full-length single strand that includes the limiting primer. This improves the efficiency of asymmetric amplification and increases the hairpin signal relative to the amplicon duplex.

The influence of hairpin loop size, stem length, and single-base mismatches within the stem were studied by PCR of well-defined plasmids *(21)*. Fig. 2 displays the effects of hairpin loop and amplicon size using the same hairpin stem. As the amplicon increases in size, the Tm of the amplicon increases as expected. However, the Tm of the hairpin decreases with increasing amplicon and loop size. Shorter loops result in more stable hairpins, and the relationship appears quantitative over the range of 17–135 bases (Fig. 2, inset). Compared with unlabeled probes of the same sequence *(15)*, snapback primers with a 34-base loop increase Tm by about 10 °C (data not shown). The intensity of the hairpin to the amplicon signal is maximal at an amplicon length of 120 bp and diminishes with longer amplicons, particularly above 300 bp.

Fig. 3A shows the effect of snapback stem length on Tm. The relative strength of the stem to amplicon signal is greater than in Fig. 2 because a 2-bp mismatch was included at the 5′ end of the snapback primers to prevent extension from the minor snapback product. The stem length was varied from 8 to 20 bp, with minimal variation in amplicon and loop lengths. Surprisingly, the signal from the 8-bp duplex is similar to that from the 20-bp duplex, although the duplex was less than half the length. Supplemental Fig. 1 shows an expanded study with duplexes 6–28 bp in length. The Tm appears linearly related to the duplex length. Single-base heterozygotes are easily distinguished from homozygotes using duplexes of either 12 or 20 bp (Fig. 3B). The A:C mismatch of the heterozygotes decreased the hairpin Tm by 6–8 °C.

The ability of snapback primers to distinguish between all single-base variants at 1 position is demonstrated in Fig. 4. Different homozygotes are compared in Fig. 4A with perfectly matched hairpins (A:T) more stable than A:G, A:A, and A:C mismatches. Furthermore, all heterozygotes (Fig. 4B) are easily identified and distinguishable. Discrimination between single-base genotypes is best when the variation is within the

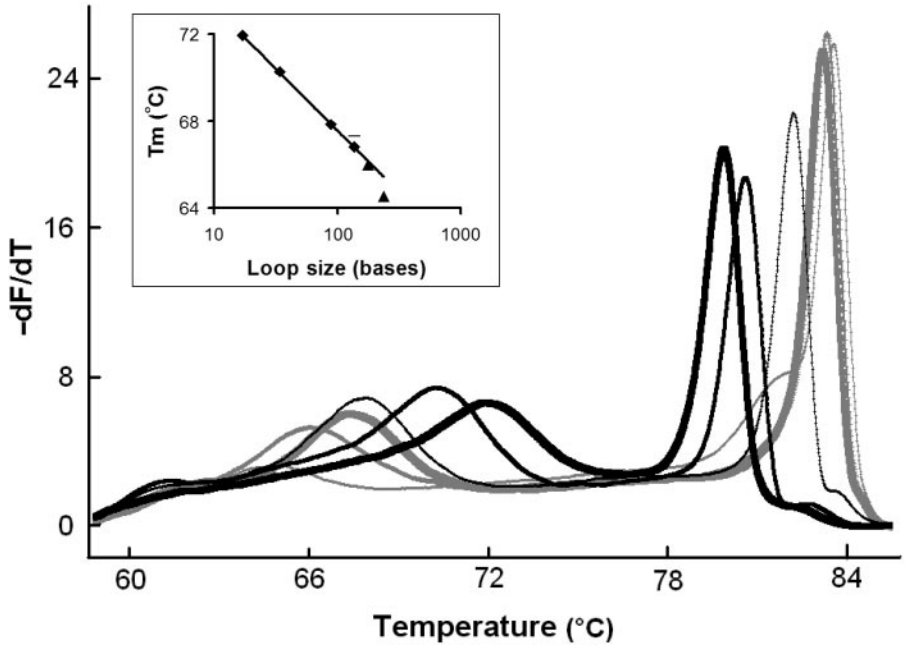

Fig. 2. The effect of loop size on snapback primer melting after asymmetric PCR.

Snapback primers were designed to anneal at different distances from the same site targeted by a common 24-bp probe element. The resulting PCR products ranged from 102 to 322 bp in size, with Tm values of 80–84 °C and hairpin loop lengths of 17–236 bases. Complete melting curves are shown, including both hairpin and amplicon melting transitions with loops lengths of 17 bases (thick black line), 34 bases (intermediate black line), 88 bases (thin black line), 135 bases (thick grey line), 177 bases (intermediate grey line), and 236 bases (thin grey line). The curves were normalized, exponential background was subtracted, and the negative first derivative of fluorescence with respect to temperature is displayed (main figure). The hairpin Tm values decreased as the loop sizes increased (inset). All hairpin duplexes were 24 base pairs in length, except for the 135-base loop where an additional complementary base resulted in a 25-bp duplex. The observed Tm of the 25-bp duplex (horizontal line) was corrected for the extra base pair stabilization by nearest-neighbor predictions *(40)* without considering loop or terminal mismatch effects. A log-linear relationship (line fit to experimental data) is apparent for loop sizes of 17–135 bases.

middle third on the stem; variants near the ends of the stem were not always detected (not shown).

Snapback genotyping from human genomic DNA is shown in Figs. 5 and 6. *F5* 1691G>A genotyping of 100 previously typed samples in 384-well format is shown in Fig. 5. The melting curves grouped into 3 obvious clusters with perfect correlation to previously established genotypes. The hairpin stem is 16 bp, and the G:T mismatch of the mutant allele decreases the Tm by 5–6 °C. The potential for more complex genotyping is shown in Fig. 6, where 7 different genotypes at 2 different loci are genotyped by 2 snapback primers and symmetric PCR. Ten-fold dilution after PCR strongly favors intramolecular snapback formation over PCR product formation. All heterozygotes resolve into 2 melting peaks except for the I506V heterozygote; however, even the I506V heterozygote is clearly distinguished from other genotypes by the shape of its melting curve after high-resolution melting analysis.

Discussion

Fluorescent melting analysis has been used since 1997 for closed-tube genotyping *(26)*. Recent advances include using unlabeled probes and saturating DNA dyes for genotyping *(15)* and high-resolution melting for variant scanning *(11, 25)*. In most cases, genotyping can be obtained by amplicon melting alone with high-resolution instrumentation and software *(27)*. However, accuracy depends on instrument resolution *(12–14)*, and even with the best instruments there is some risk of failing to differentiate certain genotypes *(25, 27, 28)*. Genotyping by probe melting is generally considered more reliable, although the inclusion of 1 or 2 labeled probes *(29, 30)* increases cost and complexity. The introduction of unlabeled probes *(15)* eliminated the need for covalently attached fluorescent dyes. The product strand complementary to the unlabeled probe is overproduced by asymmetric PCR, with 1

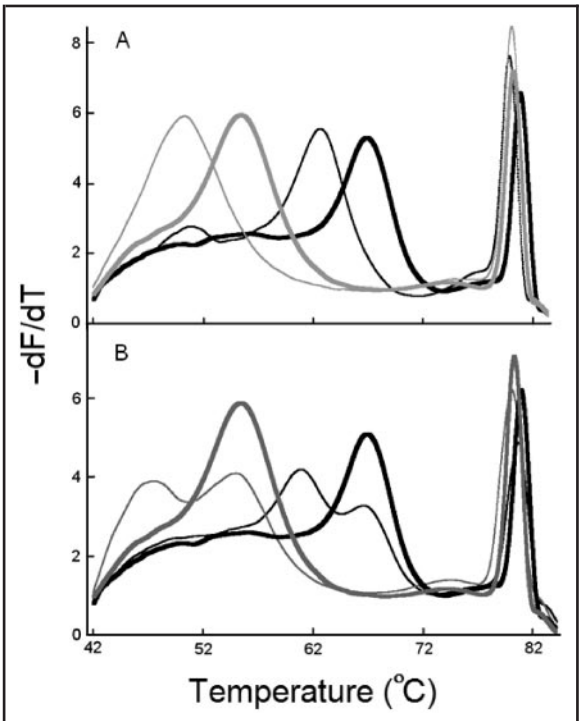

Fig. 3. Effect of hairpin duplex length (A) and genotype and hairpin duplex length (B) on melting curves.
Keeping the PCR product size (106–118 bp) and loop size (36–42 bases) approximately constant, hairpin duplex lengths were varied from 8 to 20 bp. Data are displayed as negative derivative plots after normalization and exponential background subtraction. The snapback primer tails included a 2-base mismatch at the 5′ end to prevent extension of the 3′ end of the minor product hairpin. In (A), melting curves with hairpin duplex lengths of 8 (thin grey line), 12 (thick grey line), 17 (thin black line), and 20 (thick black line) after asymmetric PCR are shown. In (B), melting curves from 12-base (grey lines) or 20-base (black lines) probe elements are shown after PCR of heterozygous A/G templates (thin lines) or homozygous A/A templates (thick lines).

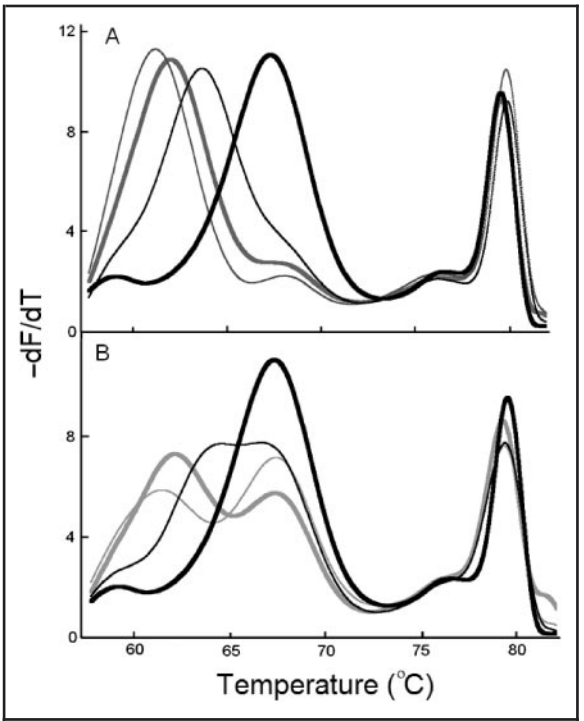

Fig. 4. Snapback primer genotyping of homozygous (A) and heterozygous (B) templates.
The snapback primer included a 24-base probe element and a 2-base 5′-end mismatch. The PCR product was 122 bp in length, and hairpins formed a 34-base loop. In (A), genotypes included A/A (thick black line), C/C (thin black line), T/T (thick grey line), and G/G (thin grey line), forming matches and A:G, A:A, and A:C mismatches in the major hairpin, respectively. In (B), the genotypes were A/A (thick black line), A/C (thin black line), A/T (thick grey line), and A/G (thin grey line).

primer in 5- to 10-fold excess. The unlabeled probe is blocked to prevent extension, requiring a nonfluorescent modification. These methods have been recently reviewed *(8, 10, 24)*.

Although there are many advantages of unlabeled probes for genotyping, there are also some disadvantages. Unlabeled probes need to be blocked at the 3′ end so that extension is prevented. The least expensive blocker is a 3′-phosphate; however, a 3′-phosphate is not very stable, and a variable proportion of the probe may retain a free 3′-OH *(17)*. This can lead to extension during PCR and the appearance of melting peaks with Tm values higher than expected. Although other blockers are more stable *(17)*, their cost is higher. Unlabeled probe genotyping requires 3 oligonucleotides: 2 primers and an additional unlabeled probe. Furthermore, unlabeled probes give the best signal when they are relatively long, usually 25–35 bases *(15)*.

Snapback genotyping, similar to snapback SSCP *(2)* and Scorpion primers *(3)*, uses a self-probing amplicon by incorporating an unlabeled probe into 1 primer as a 5′ extension. In snapback genotyping, only 2 standard oligonucleotides are necessary, 1 with a 5′ tail that snaps back to form the stem of an intramolecular hairpin. No 3′-blocking is necessary because asymmetric PCR produces 1 major hairpin with only a 5′ hybridized end. Inclusion of a terminal 2-base mismatch prevents extension from the 3′ end of the potential hairpin of the minor strand. The integrity of this

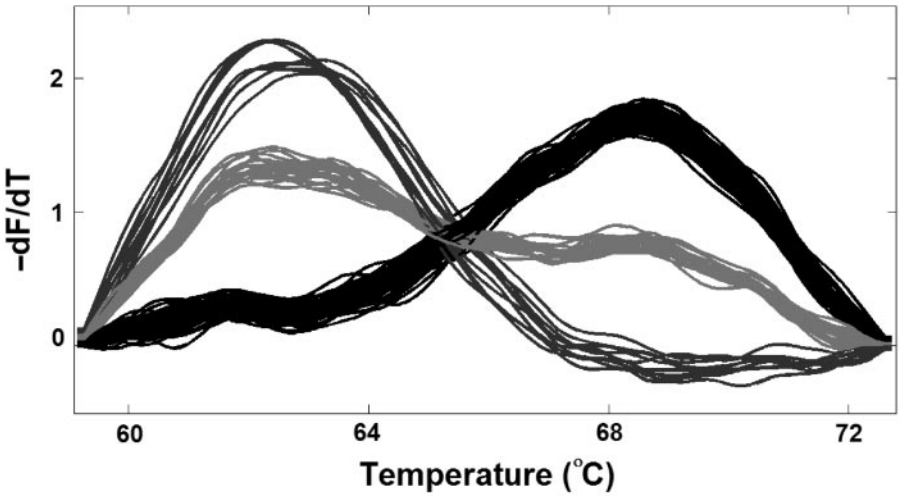

Fig. 5. *F5* **1691G>A (Leiden) genotyping with snapback primers.**

A snapback primer with a 16-base probe element and a 2-base 5′-end mismatch was used in asymmetric PCR, producing a 169-bp PCR product and a hairpin with a 99-base loop. One hundred previously typed clinical samples were PCR amplified on a 384-well plate and melted on a 384-well LightScanner. After normalization and background subtraction of the hairpin duplex region, the curves were displayed on a negative derivative plot and automatically clustered. Genotypes were 70 homozygous wild type (black), 20 heterozygous (dark grey), and 10 homozygous mutant (light grey). The probe element has a G:T mismatch to the mutant allele.

2-base mismatch can be guaranteed by eliminating termination sequences with trityl-ON column purification of the snapback primer.

With Scorpion primers, PCR is symmetric, and intramolecular hairpin formation predominates over intermolecular primer hybridization *(4)*. In snapback primer genotyping, amplification is typically asymmetric so that one strand is overproduced. However, excess snapback primer is usually present after PCR and may compete with the intramolecular hairpin. Indeed, minor shadow peaks are sometimes seen 10–15 °C below the more stable hairpin melting peaks (Fig. 2 and Fig. 4). These shadow peaks may be eliminated by using an excess of the non-snapback primer in the asymmetric PCR. In this case, the intramolecular hairpins form on the opposite strand, but the excess primer does not include a probe element, so that competition with the intramolecular hairpin is not possible. Additional minor peaks from alternative secondary structure of the amplicon may also occur. They are generally not dependent on the snapback tail and can affect both unlabeled probe and snapback primer genotyping. Such structures can be predicted by models of DNA folding *(31)* and can usually be eliminated by moving the primers to avoid the offending area.

The simplicity of only 2 unmodified oligonucleotides for probe-based genotyping is attractive, although the design of the snapback primer tail requires specific consideration. The snapback tails are usually positioned so that the variant locus is near the center of the snapback stem because sequence variants near the ends of the stem are more difficult to genotype. In contrast to reports using molecular beacons *(32)*, both the identity (Fig. 4) and position of the mismatch appear important for snapback genotyping. One potential disadvantage of snapback primers is that the primers cannot be studied independently of the probe. Separate optimization of the PCR without the probe is not possible. Nevertheless, we have used snapback primers to genotype >100 different single base variants from human genomic DNA, and have yet to find a target that could not be genotyped, although some optimization may be required.

Either 5′ exonuclease–positive or –negative polymerases can be used for snapback genotyping. The advantage of a 5′ exonuclease–positive polymerase is that hairpin annealing temperatures higher than the PCR extension temperature can be used, without concern for allele-specific amplification or PCR inhibition. The advantage of a 5′ exonuclease–negative polymerase (as used here) is that no cleavage of secondary structures is possible *(33)*. Although not usually necessary, snapback annealing during PCR can be completely avoided if the snapback Tm is kept below the PCR annealing temperature.

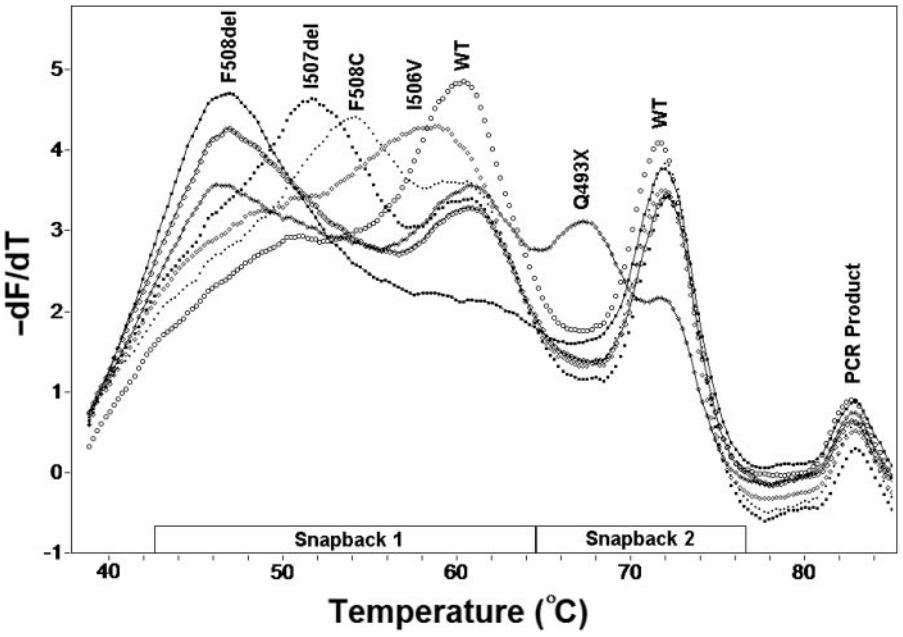

Fig. 6. Two-locus genotyping with bilateral snapback primers, symmetric PCR, and product dilution.

Primers included a 2-base 5′-end mismatch and either a 17-base (Snapback 1) or a 28-base (Snapback 2) probe element producing a 249-bp PCR product of exon 10 of *CFTR* with hairpin loops of 69 and 66 bases, respectively. Samples (2 μL) were PCR amplified on a 96-well plate, diluted 10-fold with 18 μL water, denatured, cooled, and melted on a 96-well LightScanner. Snapback 1 covered the F508del, I507del, F508C, and I506V variants with melting transitions between 46 and 60 °C. The longer snapback 2 covered the Q493X variant and melted between 66 and 72 °C. Data are displayed as a negative derivative plot after normalization and background subtraction. Samples included wild type (circles), compound F508del/Q493X heterozygote (connected small diamonds), I506V heterozygote (small diamonds), F508C heterozygote (small squares), I507del heterozygote (large squares), F508del heterozygote (connected large diamonds), and F508del homozygote (connected squares).

Intramolecular (snapback) hybridization can be favored by lowering the nucleic acid concentration. By diluting PCR products 10-fold after amplification, snapback formation is so favored that symmetric amplification can be performed and both primers can include snapback tails for interrogation of 2 separate loci (Fig. 6). However, reagent addition breaks the closed-tube system and the signal-to-noise ratio is decreased. The lower signal is not a concern with the highly sensitive instruments used here, even at their rapid melting rates of 0.15–0.3 °C/s. Performance on less sensitive instruments may be compromised, particularly since they already melt at slower rates to achieve adequate performance *(14)*. Alternatively, very rapid cooling might be used to favor hairpin formation without dilution.

Both snapback primer and unlabeled probe genotyping can be performed on common real-time instruments with variable success *(12–15)*. For example, we have performed snapback genotyping successfully on the LC480 system (Roche). However, exponential background subtraction *(24)* is not widely available. Without exponential background subtraction, the snapback melting transition can be difficult to discern, particularly before derivative plots are generated. Best performance is obtained on high-resolution systems with exponential background subtraction as demonstrated here.

At least 3 factors increase the observed Tm of snapback hairpins and enable the use of short stem lengths for genotyping. First, intramolecular hybridization stabilizes the hairpin several degrees over the corresponding unlabeled probe. Shorter loops of 15–20 bases are stabilized more than longer loops of 200–350 bases (Fig. 2), a nonlinear correlation previously observed *(34)*. Loop sizes formed by snapback primers generally cannot be smaller than 15–20 bases (the portion of the snapback primer that initially anneals to the target). Second, hairpin Tm under PCR conditions appears linearly related to the stem duplex length (Supplemental Fig. 1). In contrast, the Tm of intermolecular probes decreases much more rapidly as the duplex length gets

smaller. Third, the presence of the dye, LCGreen Plus, increases Tm by 2–4 °C *(15)*. Hairpin stability is also very sensitive to ionic strength (particularly Mg^{2+}) *(35)*. Uncertainties introduced by the dye, ionic stabilization, and the hairpin loop make precise Tm predictions difficult. Nevertheless, the overall effect is that under PCR conditions with saturation dyes, small hairpin duplexes are stabilized. In some diagnostic assays, short probes are preferred because of extensive sequence diversity. In such cases, snapback genotyping provides an alternative strategy to the use of minor groove binders *(36)*, locked nucleic acids *(37)*, and peptide nucleic acids *(38)*.

Snapback primers are an interesting option for simple, closed-tube, single-color genotyping. They provide the specificity of a probe with only 2 unmodified oligonucleotides. Real-time PCR is not required. However, the ability to distinguish genotypes that differ only slightly in stability depends on the melting curve resolution of the instrument *(12–14)*, and analysis is best enabled by exponential background subtraction of the melting curve *(24)*. Together with unlabeled probes *(15)*, small amplicons *(27)*, and allele-specific methods *(39)*, single-color genotyping by melting takes the complexity out of the oligonucleotide reagents by eliminating covalently attached fluorescent dyes.

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