Reports

Rare allele enrichment and detection by allele-specific PCR, competitive probe blocking, and melting analysis

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Differential amplification of variant and wild-type alleles by PCR is often used for rare allele enrichment. We have combined allele-specific PCR, competitive probe blocking, asymmetric PCR, and melting analysis to enhance rare allele detection in a homogeneous system. Unlabeled, dual hybridization or molecular beacon probes were used for competitive blocking of the wild-type allele at a concentration 10 times that of the allele-specific primer. In each case, rare alleles were detected by probe melting analysis at a sensitivity of >0.001% (1 variant copy within 100,000 wild-type copies), providing single copy detection in typical PCRs. Ninety-one thyroid biopsies were tested for the *BRAF* mutation p.V600E (c.1799 T>A) by both dual hybridization probes without enrichment and an allele-specific, competitive blocking melting analysis with unlabeled probes. Eighty-seven samples were concordant between methods (43 positive, 44 negative), while 4 samples that were negative by direct analysis became positive after enrichment. Probes that both block wild-type amplification and detect rare variants by melting analysis improve the detection sensitivity of allele-specific PCR for rare alleles. In particular, melting analysis using unlabeled probes and amplification by rapid-cycle PCR provides cost-effective and fast enrichment and detection of rare alleles.

The ability to detect low levels of sequence variants among mostly wild-type DNA is critical for early cancer detection, prenatal testing, and infectious diseases. In cancer, low-level mutations (<10%) are usually below the detection limits of most genotyping techniques, including standard sequencing. Many moderate- to high-sensitivity PCR-based methods have been developed over the past two decades to enrich minority alleles. Limiting the denaturation temperature of PCR can be used to enrich known and unknown mutations (1). However, careful control of cycling temperatures is necessary and multiple amplicons can seldom be analyzed on the same plate. Allele-specific PCR methods to enrich known mutations include the amplification refractory mutation system (2), mutant allele-specific amplification (3), and methods that incorporate locked nucleic acids for increased specificity (4,5). Alternatively, nucleic acid clamps can be used to block wild-type amplification, including peptide nucleic acid (6–9) and locked nucleic acid (10–12) probes. In some methods, primers that select mutant DNA are combined with probes that block wild-type amplification. In one version, the blocking

probe competes with the allele-specific primer (13,14) for increased sensitivity. However, all these methods can result in false positives when sensitivities below 0.1% are attempted (13–15).

Herein we enhance allele-specific PCR with a competitive blocking probe by asymmetric amplification and probe melting analysis to increase sensitivity and specificity. Unlabeled probes (16,17), dual-hybridization probes (18,19), and molecular beacons (20) are used as probes and also identify rare alleles by melting analysis. The method is demonstrated on the *BRAF* point mutation p.V600E (c.1799 T>A), the most common change in papillary thyroid carcinoma (21) that occurs in more than 80% of cases studied (21,22).

Materials and methods

DNA samples

DNA from normal human blood and a homozygous *BRAF* c.1799T>A cell line (Cat. no. HTB-72; ATCC, Manassas, VA, USA) were extracted by salting out (Gentra, Puregene, Qiagen, Valencia, CA, USA). DNA concentrations and quality were assessed by absorbance (NanoDrop, Wilmington, DE,

USA) with minor adjustments by PCR quantification cycle (C_q). Different mutation percentages (10%, 1%, 0.1%, 0.01%, 0.001%, and 0.0001%) were obtained by mixing.

Clinical samples were obtained from a total of 47 patients. Forty-four of these patients had samples with both surgical excision tissue and fine-needle aspirates of thyroid nodules while the remaining 3 patients had only needle aspirates. All samples were de-identified and blinded by ARUP Laboratories (Salt Lake City, UT, USA) after testing for *BRAF* c.1799A using dual-hybridization probes as described previously (23).

Oligonucleotides

The allele-specific primer 5'-GTGATTTT-GGTCTAGCTACAGA-3', reverse primer 5'-TCAGTGGAAAAATAGC-CTCAATTC-3' and unlabeled probe 5'-TCTAGCTACAGT-GAAATCTCGATG-P-3' were synthesized at the University of Utah core facility by standard phosphoramidite synthesis. Dual-hybridization probes 5'-AGCTACAGT-GAAATCTCGATGGAG-fluorescein-3' and 5'-LCRed640-GGTCCCAT-CAGTTTGAACAGTTGTCTGGA-

Figure 1. Derivative melting curves obtained after PCR in the presence of unlabeled blocking probes that detect the *BRAF* c.1799T>A mutation. The melting curve resulting from 100% wild-type DNA is shown in black, while the 100% variant DNA is shown in gray. The thin black line is the no-template control. For both DNA templates, the resulting amplicon peak is at 83°C. The melting peak of the probe/amplicon duplex is 66°C for the matched wild-type allele and 62°C for the mismatched mutant allele. The detection sensitivity for the 1799A variant depends on the annealing/extension temperature during PCR. Sensitivity is maximal when the annealing/extension temperature is between the T_m s of the wild-type and mutant duplex peaks (top). Supporting data for the indicated sensitivities are shown in Supplementary Figure S1.

P-3' were synthesized by Idaho Technology (Salt Lake City, UT, USA). The molecular beacon 5'-FAM-CGGTCTAGCTA-CAGTGAATCTCGACCG-BHQ1-3' was synthesized by Biosearch Technologies, Novato, CA, USA). The underlined bases are matched to the c.1799T>A mutation, and "P" signifies a 3'-phosphate.

PCR and melting analysis

PCR was performed in 10- μ L reactions containing 2 mmol/L $MgCl_2$, 50 mmol/L Tris (pH 8.3), 500 mg/L BSA, 200 μ mol/L of each dNTP, 0.4 units KlenTaq polymerase (Ab Peptides, St. Louis, MO, USA), 64 ng/ μ L Anti-Taq monoclonal antibody (eNZYME, Montgomery Village, MD, USA), 0.5 $\times$ LCGreen Plus (Idaho Technology), and 50 or 500 ng human genomic DNA. For symmetric PCR, both primers were at 0.5 μ M. For asymmetric PCR, the allele-specific primer was reduced to 0.1 μ M or 0.05 μ M. In experiments with blocking probes, the allele-specific primer was at 0.05 μ M with a probe concentration of 0.5 μ M.

Eighty cycles of rapid-cycle PCR was performed on a LightCycler 1.5 (Roche Applied Science, Indianapolis, IN, USA) after an initial denaturation 95°C for 1 min, unless otherwise specified. When an unlabeled probe or no probes were used, each cycle consisted of a 95°C denaturation for 0 s and a 64°C annealing/extension phase for 4 s with programmed transitions of 20°C/s. Following PCR, a melting protocol from 55°C to 92°C with a 0.2°C/s ramp rate was used. The protocols for dual hybridization and molecular beacon probes were similar except the annealing/extension phase of PCR was

64°C for 0 s and a 0.1°C/s ramp between 50°C and 75°C was used for melting. The second derivative method (24) was used to determine quantification cycle (C_q) values (25).

Single-copy DNA amplification

Digital PCR was performed by diluting *BRAF* c.1799A genomic DNA to less than one copy per reaction in the presence or absence of wild-type DNA. Approximately 48 copies (0.144 ng) of c.1799A DNA were amplified across the 96 wells of the LC480 (Roche) after an initial denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 64°C for 15 s. In a separate experiment, ~10 copies (0.030 ng) of c.1799A DNA admixed with 3.2 $\times$ 10⁶ copies (10 μ g) wild-type genomic DNA (0.0003% mutant DNA) were amplified after separation into 20 reactions on a LightCycler 1.5, using the unlabeled probe cycling conditions.

Results and discussion

Allele-specific PCR is widely used to enrich rare alleles. Specificity is obtained by matching the 3'-end of one primer to the desired allele. Other alleles are mismatched and their amplifications are inhibited. Inhibition is not complete, however, and mismatched alleles are amplified to various extents depending on the type of mismatch (26,27). The lowest rare-allele percentage that can be distinguished from pure wild-type DNA establishes the sensitivity of the method and is conveniently studied by real-time PCR. Lowering the Mg^{2+} and the primer concentrations can increase sensitivity and lower the false-positive rate (28).

We enhanced allele-specific PCR amplification and detection by using asymmetric PCR, a wild-type blocking probe, and probe melting analysis. Rare allele enrichment was optimal with an excess of blocking probe and reverse primer compared with the allele-specific primer. Using the *BRAF* c.1799A single base mutation, as the concentration of the allele-specific primer decreased from 0.5 μ M to 0.1–0.05 μ M (in the absence of blocking probes), specificity increased while PCR efficiency decreased. Increasing specificity was reflected during real-time PCR by the ΔC_q between wild-type and *BRAF* DNA increasing from 10 to 19–20 cycles. Decreasing PCR efficiency was evidenced by an increase in the C_q of *BRAF* DNA by 12–15 cycles. Although PCR efficiency was affected by decreasing the concentration of either primer, only lower concentrations of the allele-specific primer increased specificity. In order to compensate for the lower PCR efficiency, 80 cycles were typically performed, but this required only 30 min using rapid-cycle PCR (29).

A lower concentration of allele-specific primer produces an excess of one product strand during asymmetric PCR. If melting probes complementary to the excess strand are included, melting signatures can be observed that are specific to the allele under the probe (16). Figure 1 displays derivative melting plots obtained after asymmetric PCR of pure wild-type or *BRAF* c.1799A DNA using an unlabeled wild-type blocker as the melting probe. The matched wild-type allele melts about 4°C higher than the mismatched mutant allele. Similar to allele-specific competitive blocker PCR (13), the wild-type probe preferentially binds to wild-type DNA and competes with primer binding. At the same time, the allele-specific primer is matched to and preferentially extends the rare allele. Both allele-specific extension and wild-type blocking enhance enrichment and sensitivity for detecting the rare allele.

Allele-specific enrichment with a blocking probe is affected by the annealing/extension temperature of rapid-cycle, two-step PCR. Specificity is optimal when the annealing/extension temperature is between the melting temperatures (T_m) of the wild-type blocking probe for the matched wild-type allele and the mismatched mutation allele. As the annealing/extension temperature was varied from 60°C to 68°C, detection sensitivity reached a maximum of 0.001% at 64°C (Figure 1, top and Supplementary Figure S1, panel 3), between the two peaks where discrimination between wild-type and mutant DNA is greatest. If the annealing temperature is lower than or equal to the T_m of the mutant allele (62°C), the probe suppresses amplification of both wild-type

Figure 2. Real-time PCR (A) and melting analysis (B–D) of allele-specific, competitive blocking, asymmetric PCR with an unlabeled probe in the presence of 50 ng of total DNA. In panels A and B, the percentage of c.1799A DNA is 10% (thick solid line), 1% (large dots), 0.1% (squares), 0.01% (dashes), and 0.001% (dots). The gray line is the no-template control and the thin black line is pure wild-type DNA. The apparent amplification of the 0.001% sample by real-time PCR is clearly nonspecific when analyzed by melting. The unlabeled probe melting peak for the 1799A allele is at 62°C and the intended PCR product peak is at 83°C. In panels C and D, 100% mutant DNA (thick black), 100% wild-type DNA (intermediate black), and no-template DNA (thick gray) controls are shown. The thin gray lines are 10 replicates of 1.7 copies of mutant DNA per tube in panel C, or 0.17 copies of mutant DNA per tube in panel D.

and mutant alleles, limiting the sensitivity to 0.1%–0.01% (Supplementary Figure S1, panels 1 and 2). If the annealing temperature is higher than or equal to the T_m of the wild-type allele (66°C), preferential blockage and PCR efficiency decrease, also limiting sensitivity to 0.01% (Supplementary Figure S1, panels 4 and 5). Although rapid-cycle PCR was usually performed, similar results were obtained on more conventional plate instruments. This is in contrast to rare allele detection with snapback primers, where rapid cycling was critical (30).

Using optimal oligonucleotide concentrations and cycling conditions, the sensitivity of the asymmetric melting method was determined with and without wild-type blocking probes. With no probe, the sensitivity determined from the real-time curves was 0.1% (data not shown). This is similar to other reports using allele-specific primers (31). Sensitivity increased ≥ 10 -fold when an unlabeled probe blocked the wild-type allele and 50 ng (1.7×10^4) total genomic DNA was present (Figure 2). Although allele-specific PCR with competitive blocking greatly reduces amplification of wild-type DNA, false positives may still occur when real-time PCR is used without melting analysis. In Figure 2A, the 0.001% sample appears to amplify at high cycles. However, both the probe peak and the target amplicon ($T_m = 83^\circ\text{C}$) are missing for this sample and a different undesired product is present ($T_m = 75^\circ\text{C}$), indicating nonspe-

cific amplification (Figure 2B). By melting the blocking probe, the mutant melting peak confirms that 0.01% mutant DNA (1.7 copies/tube) was amplified, while 0.001% (0.17 copies/tube) was not. To further investigate the reproducibility of these results, 10 tubes were amplified and melted with an average of 1.7 mutant copies per tube (Figure 2C). Nine of the 10 tubes clearly showed an unlabeled probe melting peak, while one tube did not. This is close to the expected stochastic result predicted by the Poisson equation where ≥ 1 copy should be present 82% of the time, with zero copies present 18% of the time. Similarly, when 10 tubes were amplified with an average of 0.17 copies per tube, 9 had no evidence of a probe peak while 1 clearly was positive (Figure 2D). This is also very close to the Poisson prediction of 16% of samples containing ≥ 1 copy and 84% with 0 copies.

Rare allele detection by probe melting analysis is more specific than real time PCR because the T_m of the probe/allele hybrid confirms the presence of the mutant allele. In contrast, real-time PCR without probes cannot distinguish the intended PCR product from primer dimers and other nonspecific PCR products. The method is enabled by hybridization probes that provide a melting signature. Hydrolysis probes cannot be used because the competition between the probe blocker and primer does not allow probe hydrolysis. Labeled hybridization probes like dual-hybridization probes

and molecular beacons have the potential for multicolor analysis. However, unlabeled probes have the advantages of low cost and additional confirmation of the correct product by the amplicon melting peak.

To assess the potential for even greater sensitivity, samples with different mutant percentages were amplified in a total of 500 ng (1.7×10^5 copies) DNA (Figure 3). Dual hybridization probes (Figure 3B) and molecular beacons (Figure 3C) were designed to anneal to the same region as the unlabeled probe design (Figure 3A), each complementary to the wild-type sequence with the same T_m . Unlabeled probes, dual-hybridization probes, and molecular beacons all had a sensitivity of $\geq 0.001\%$ when used as blocking probes. Samples with 0.001% mutant DNA (1.7 copies/tube) had identifiable probe melting peaks of the mutant allele, while samples with 0.0001% mutant DNA (0.17 copies/tube) did not.

To verify that the method detects the presence or absence of a single template copy, 48 copies of genomic *BRAF* mutant DNA were distributed across the wells of a 96-well plate and amplified. The melting curves clearly clustered into 2 groups with 43 positive and 53 negative results (data not shown). The ability of melting analysis to distinguish the presence or absence of single-copy templates without probes has been previously reported (32). Similar results have been reported for digital PCR with labeled probes, where the complexity of the genomic background is limited (33). However, specific amplification becomes more difficult as the concentration of background wild-type DNA increases. Nevertheless, when 10 copies of *BRAF* mutant DNA were admixed with 10 μg wild-type DNA (3.4×10^6 copies) and amplified in 20 PCR capillaries, 7 out of 20 reactions were positive by melting analysis. Even in the presence of $>10^5$ copies of wild-type DNA, single mutant DNA molecules were easily distinguished from the complete absence of mutant DNA. The sensitivity may even be greater, but testing is difficult because of the high DNA concentrations and/or PCR volumes required. If a mutant copy is present in the sample, it will be detected without the risk of false-positive reactions.

Increased sensitivity was demonstrated on 91 clinical thyroid samples previously tested for *BRAF* c.T1799A (43 positive, 48 negative) by dual-hybridization probes (30). These samples were blinded and reanalyzed by the unlabeled probe method described above. All samples were concordant except for an additional four samples that were positive by the allele-specific method. Detection of *BRAF* c.T1799A increased in these clinical samples by 9.3% when the allele-specific, competitive blocking method with melting was used.

Figure 3. Derivative melting curves of allele-specific, competitive blocking, asymmetric PCR. (A) Unlabeled probes, (B) dual-hybridization probes, and (C) molecular beacons. The percentage of c.1799T>A DNA in 500 ng total DNA is: 1% (thick solid line), 0.1% (large dots), 0.01% (squares), 0.001% (dashes), and 0.0001% (small dots). The gray line is the no-template control and the thin black line is 50 ng pure wild-type DNA. Probe- and allele-specific primer schematics are shown in each panel.

The enrichment method used here is similar to allele-specific competitive blocker PCR that is typically analyzed on agarose gels (13) or by internal hydrolysis probes (15). However, the blocker oligonucleotide serves a dual purpose: both blocking wild-type DNA and indicating the presence of mutant alleles by melting analysis. Asymmetric PCR is employed to increase allele-specific amplification and augment detection of the melting signal. Detection of the mutant allele by melting prevents false-positive results, even with $>10^5$ -fold excess of wild-type DNA. Unlabeled, dual hybridization, and molecular beacon probes can all function as blockers with similar sensitivities. Unlabeled probes are low in cost, while dual hybridization and molecular beacon probes can be multiplexed by color. The method is fast, performed in a closed tube, and should work on any thermocycler with melting analysis capability. The combination of allele-specific priming, competitive probe blocking of wild-type amplification, asymmetric PCR, and probe melting analysis allows single copy detection of variant alleles down to a sensitivity of $\geq 0.001\%$ mutant to wild-type DNA.

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Competing interests

Aspects of melting analysis, unlabeled probes and dual-hybridization probes are licensed from the University of Utah to Idaho Technology. C.T.W. has equity interest in Idaho Technology.

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