Enrichment and Detection of Rare Alleles by Means of Snapback Primers and Rapid-Cycle PCR

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BACKGROUND: Selective amplification of minority alleles is often necessary to detect cancer mutations in clinical samples.

METHODS: Minor-allele enrichment and detection were performed with snapback primers in the presence of a saturating DNA dye within a closed tube. A 5′ tail of nucleotides on 1 PCR primer hybridizes to the variable locus of its extension product to produce a hairpin that selectively enriches mismatched alleles. Genotyping performed after rapid-cycle PCR by melting of the secondary structure identifies different variants by the hairpin melting temperature (\(T_m\)). Needle aspirates of thyroid tissue (n = 47) and paraffin-embedded biopsy samples (n = 44) were analyzed for BRAF (v-raf murine sarcoma viral oncogene homolog B1) variant p.V600E, and the results were compared with those for dual hybridization probe analysis. Needle aspirates of lung tumors (n = 8) were analyzed for EGFR [epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)] exon 19 in-frame deletions.

RESULTS: Use of 18-s cycles and momentary extension times of \(0.9\) s with rapid-cycle PCR increased the selective amplification of mismatched alleles. A low Mg\(^{2+}\) concentration and a higher hairpin \(T_m\) relative to the extension temperature also improved the detection limit of mismatched alleles. The detection limit was 0.1% for BRAF p.V600E and 0.02% for EGFR exon 19 in-frame deletions. Snapback and dual hybridization probe methods for allele quantification of the thyroid samples correlated well (\(R^2 = 0.93\)) with 2 more BRAF mutations (45 and 43, respectively, of 91 samples) detected after snapback enrichment. Different EGFR in-frame deletions in the lung samples produced different hairpin \(T_m\)s.

CONCLUSIONS: Use of snapback primers for enrichment and detection of minority alleles is simple, is inexpensive to perform, and can be completed in a closed tube in <25 min.

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Since the sequencing of the human genome and the development of high-throughput genotyping techniques, many genes that are associated with disease through single-base and other mutations have been identified. For example, both prenatal and cancer diagnostics previously performed by chromosomal or phenotype analysis are now done at the nucleotide level (1–3). Mutations in DNA circulating in the plasma can serve as biomarkers of early tumor development and the potential response to treatment (4, 5). A somatic mutation of the BRAF\(^v\) (v-raf murine sarcoma viral oncogene homolog B1) gene, c.1799 T>A, which encodes the substitution p.V600E, is the most common change in papillary thyroid carcinoma (6), having occurred in >80% of the cases studied (7). In lung cancer, in-frame deletions of exon 19 in the EGFR gene [epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)], which encodes the epidermal growth factor receptor, can confer either a positive therapeutic response to tyrosine kinase inhibitors or drug resistance (8, 9).

In the early and posttreatment stages of cancer, the proportions of mutant alleles in clinical samples are typically low compared with the wild-type allele. Even after amplification by standard PCR, concentrations of the minority allele may not be sufficient to permit genotyping or sequencing (10–13). There is a wide range of PCR-based techniques for enriching the proportion of minority alleles and mutations in a sample. When the genotype of the mutation is unknown,
COLD PCR (coamplification at lower denaturation temperature PCR) can be used (14). This technique can detect 1% of a mutant allele, but additional analysis is necessary to identify the variant. Some of the most popular techniques for enriching known mutations are ARMS (amplification refractory mutation system) (15), peptide nucleic acid (PNA)-mediated PCR (16, 17), locked nucleic acid (LNA)-mediated wild type–blocking (WTB) PCR (18), MAMA (mismatch amplification mutation assay) PCR (19), TaqMAMA (20, 21), and use of Scorpion® primers (22). These methods detect mutations by allele-specific PCR and note differences in quantification cycle, and can detect 0.1% of mutant alleles. Except for ARMS, these methods also require fluorescently labeled oligonucleotides or specially modified primers.

A snapback primer combines a standard primer with an oligonucleotide tail that “snaps back” to hybridize with its extension product, creating a hairpin loop in the single-stranded product. A saturating dye that binds to double-stranded DNA and high-resolution melting analysis are typically used to monitor hybridization of the hairpin (23). The data are often presented in negative-derivative plots, which display the alleles as peaks. We recently noticed allele bias (different peak heights) when genotyping heterozygotes with snapback primers. A similar disparity in peak heights has previously been noted with dual hybridization probes (24), single hybridization probes (25), and unlabeled probes (26). The effect seemed particularly acute with snapback primers, however, and extreme allele bias sometimes caused heterozygotes to be incorrectly genotyped as homozygotes. Although such a bias is a disadvantage for genotyping, it can also be viewed as an advantage if applied to selective amplification and detection of rare disease alleles. In this report, we define the reaction parameters that affect allele bias with the use of snapback primers and apply them to the enrichment and detection of BRAF p.V600E and EGFR in-frame deletions.

Materials and Methods

CONTROL HUMAN GENOMIC DNA AND CELL LINES

Wild-type human genomic DNA was extracted from human blood. The BRAF p.V600E homozygous mutation in cell line HTB-72 and the EGFR homozygous deletion p.deIE746-A750 (Genebank Acc. # EAW50962) in cell line CRL-5883 were obtained from the ATCC. Genomic DNA was extracted (Gentra Puregene Cell Kit; Qiagen), quantified by spectrophotometry (NanoDrop 1000; Thermo Scientific) at A260, and with verification by quantitative PCR. Different ratios of alleles were obtained by mixing wild-type DNA and mutant DNA.

CLINICAL SAMPLES

 Archived and deidentified samples from 47 patients with thyroid nodules and from 8 patients with lung tumors were retrieved from ARUP Laboratories (University of Utah IRB 00013005). For the thyroid tumors, fine-needle aspirates stained with Diff-Quik (Dade Behring/Siemens) and aniline blue–stained, formalin-fixed, paraffin-embedded slides were available from 44 patients, whereas only needle aspirates were available from an additional 3 patients. Needle biopsies of the lung tumors were fixed on slides with triarylmethane dye dissolved in methanol. Slides were screened by pathologists for tumor selection, and DNA was prepared as previously described (27).

PRIMERS

To study enrichment reaction parameters, we used the biallelic variant rs149041370 (A/G) with primer agct tgttttcctttacttacctcacag and snapback primer ccAAGA TCACTGTGCTGtcgcttggtccacaccagga, to produce a 133-bp amplicon. For the BRAF p.V600E mutation, we used primers tgttttcctttacttacctcacag and tcagcagaaaaatagcctcaattc for regular amplification (183-bp amplicon). We replaced the latter primer with snapback primer acCTAGCTACGTGAAATCTC GATcgtggaaaaatatgctcattc for selective amplification. To detect EGFR exon 19 deletions, we used primer agct tgttttcctttacttacctcag and snapback primer ccAAGA GAAGCAACATCTCCGAAAGacgacagaactcactcagagga to produce a 131-bp amplicon. For these snapback primers, the bases in uppercase signify the probe element, and the 2 bases in lowercase at the 5’ end are mismatched to the template (23). The sequences of additional primers and dual hybridization probes for BRAF p.V600E have previously been published (27). All primers were synthesized by the University of Utah core facility. Dual hybridization probes were synthesized by Idaho Technology.

PCR AND MELTING PROTOCOLS

PCR was performed in 10-μL reaction volumes containing 0.4 U of the 5’-exonuclease negative KlenTaq™ polymerase (Ab Peptides) or 1 U of the 5’-exonuclease positive FastStart Taq DNA Polymerase (Roche), 1.6–3.0 mmol/L MgCl2, 50 mmol/L Tris (pH 8.3), 500 mg/mL BSA, 200 μmol/L of each deoxynucleoside triphosphate (dNTP), 64 ng Anti-Taq

[Nonstandard abbreviations: COLD PCR, coamplification at lower denaturation temperature PCR; ARMS, amplification refractory mutation system; PNA, peptide nucleic acid; LNA, locked nucleic acid; WTB, wild type–blocking; MAMA, mismatch amplification mutation assay; dNTP, deoxynucleoside triphosphate; Tm, melting temperature.]
Monoclonal Antibody (eENZYME), 0.5 × LCGreen® Plus (Idaho Technology), 0.5 μmol/L of the snapback primer, 0.1 μmol/L of the other primer, and 50 ng human genomic DNA. The PCR was performed in a LightCycler® (version 1.5; Roche). We used variant rs149041370 to study the effects of PCR cycling conditions and MgCl2 on rare-allele enrichment. The PCR protocol for rs149041370 included an initial denaturation step (95 °C for 20 s with KlenTaq1 or 95 °C for 10 min with FastStart Taq) followed by 70 cycles of 95 °C (0-s hold), annealing at 63 °C (0-s hold), and extension at 68 °C or 72 °C (hold of 0–20 s). The PCR protocol for the BRAF p.V600E mutation was denaturation at 95 °C for 20 s followed by 70 cycles of 95 °C for 0 s, 55 °C for 0 s, and 64 °C for 0 s. The PCR protocol for small EGFR deletions on exon 19 was the same as for BRAF except that a 68 °C extension temperature was used. Snapback PCR amplification required only 21 min, whereas the PCR protocol for the dual hybridization probe required 45 min, as has previously been described (26).

After the PCR, the capillary samples were removed from the LightCycler, placed in the high-resolution melting instrument (HR-1; Idaho Technology), and melted from 60 °C to 92 °C with a 0.5 °C/s ramping rate.

**CALCULATION OF ALLELE FRACTIONS**

Melting curves were normalized by exponential background subtraction and differentiated with Savitzky–Golay fitting (28). Allele fractions were calculated from the weighted peak heights (Fig. 1). Specifically, \(D_w(T)\) is the negative derivative of the normalized melting curve of a wild-type sample, \(D_m(T)\) is the negative derivative of the normalized melting curve of a homzygous mutant sample, and \(D_L(T)\) is the negative derivative of the normalized melting curve of a fractional mixture of the 2 samples. If the snapback probe element matches the wild-type allele, \(D_m(T)\) will have a peak at a low temperature, \(T_L\), and \(D_w(T)\) will have a peak at a high temperature, \(T_H\), with \(T_L < T_H\). \(D_L(T)\) typically exhibits 2 peaks, corresponding to melting of the mismatched allele at \(T_L\) and the matched allele at \(T_H\). The mutant allele fraction is calculated as the weighted mean of 2 estimates as: \(F_m = w_L \cdot f(T_L) + w_H \cdot f(T_H)\), where \(w_L\) and \(w_H\) are the weights, and \(f(T_L)\) and \(f(T_H)\) are the individual estimates at each temperature peak. The weights are determined by the relative magnitudes of the mixed sample above the baselines of the unmixed samples to favor the larger peak: \(w_L = a/(a + b)\), and \(w_H = b/(a + b)\). The individual estimates \(f(T_L)\) and \(f(T_H)\) are obtained proportionally at each temperature; \(f(T_L) = a/d\), and \(f(T_H) = c/e\). Therefore, \(F_m = (a^2 e + bcd)/de(a + b)\).

**SEQUENCING**

Sequencing was performed by fluorescent dideoxynucleotide termination at the University of Utah core facility. Samples that were positive for BRAF p.V600E by snapback analysis but not by hybridization probe melting were confirmed by sequencing the snapback primer–amplified product. Products amplified from mixtures containing p.V600E DNA from 50% down to 0.1% were also sequenced after both regular and snapback PCR. EGFR exon 19 deletions were confirmed by sequencing.

**Results**

Fig. 2 summarizes the probable mechanism of allele enrichment by snapback primers. The snapback probe element was mismatched to the mutation allele, destabilizing the hairpin and allowing the polymerase to unfold the secondary structure and complete the extension of the full-length PCR product (Fig. 2, left). The snapback probe element was completely matched to the wild-type allele, however, blocking extension with a more stable hairpin and limiting formation of the full-length PCR product (Fig. 2, right). Because differential amplification depends on the relative stability of the hairpin produced from a single mismatch, successful
enrichment is likely to depend on amplification conditions, including the displacement activity of the polymerase, the extension temperature relative to the hairpin stability, and the extension time.

Fig. 3 demonstrates that allele enrichment in snapback primer genotyping depends on the melting temperature \((T_m)\) and the length of the probe element. We used genomic DNA heterozygous at rs149041370 to study the effect of these parameters during PCR at an extension temperature of 72 °C. With a 9-bp probe element \((T_m, 64 °C)\), the heterozygous genotype appeared symmetric (Fig. 3A); however, asymmetry appeared as we increased the probe element length to 13 bp with a \(T_m\) of 68 °C (Fig. 3B). With a probe element length of 17 bp and a \(T_m\) of 74 °C, the wild-type peak was decreased to the extent that the genotype might be considered homozygous (Fig. 3C). Allele enrichment also depended on the extension temperature relative to the probe element \(T_m\), with lower extension temperatures showing a greater degree of enrichment (data not shown).

Allele enrichment during snapback PCR depended strongly on extension time and \(Mg^{2+}\) concentration (Fig. 4). Fig. 4A shows that shorter extension times increased enrichment of the mismatched allele. The use of lower free \(Mg^{2+}\) concentrations (after accounting for stoichiometric chelation with dNTPs) also strongly enriched the rare allele, mutant fractions as low as 0.1% were easily detected (Fig. 4B). There were no significant effects of denaturation/annealing temperatures or times on allele enrichment (data not shown). Unless otherwise specified, we obtained all data with a 5’-exonuclease–negative polymerase. When we used a 5’-exonuclease–positive polymerase, we observed similar effects on allele enrichment, although the degree of enrichment was not as great as with an exonuclease-negative polymerase (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol56/issue5).

The Braf p.V600E variant was amplified in mixtures containing 50%, 10%, 2%, 1%, and 0.1% and subsequently sequenced. Sequencing detection limits were about 10% with the regular PCR and <1% after snapback allele enrichment (see Fig. 2 in the online Data Supplement).

We amplified and analyzed mixtures of the Braf p.V600E variant and wild type at DNA percentages varying from 100% down to 0.024% with both dual hybridization probes and snapback enrichment and detection (Fig. 5). With dual hybridization probes, the observed mutation fraction reflected the actual mutation fraction (Fig. 5A), with a detection limit of about 10%. With snapback primers, the apparent mutation fraction was much higher than the actual mutation fraction, with a detection limit of approximately 0.1%. The CV \((n = 10 \text{ at each point})\) increased as the fraction of the minor allele decreased (16% at 0.1, 18% at 0.01, and 47% at 0.001).
We analyzed biopsies of thyroid masses and fine-needle aspirates for the BRAF variant with both dual hybridization probes and snapback primer enrichment. After the blinded analysis, all samples were concordant (45 positive and 46 negative) except for 2 samples that were positive by snapback primers but negative by hybridization probes. Sequencing of the snapback-amplified products confirmed the presence of the variant allele. Fig. 5B shows the allele fractions calculated from the calibration curves (Fig. 5A) for dual hybridization probes and snapback primers.

Snapback enrichment of EGFR exon 19 deletions used a 23-bp probe element to overlap the most common deletions on exon 19 (Fig. 6). Use of a 15-bp deletion (delE746-A750) as the positive control gave a $T_m$ of 67 °C, 11 °C below the $T_m$ for the wild-type allele (78 °C). The 0.02% variant mixture was enriched to a mean of 23.9% (SD, 1.2%) ($n = 10$) after the PCR, easily distinguishable from pure wild type (mean, 0.10% (2.6%); $n = 10$; $P < 10^{-5}$, $t$-test); however, the mutant allele was not detected when diluted to 0.01% under the conditions tested (see Fig. 3 in the online Data Supplement). Deletions were detected in 2 of the 8 tested lung needle biopsies. Sequencing identified both deletions as delL747-P753insS. Because the probe element hybridized to only 6 bp after this deletion, the $T_m$ of the deletion allele (52 °C) was 26 °C lower than that of the wild-type allele.

The limit of detection after snapback primer enrichment varied with the target and depended on the absolute concentration of the minor allele analyzed. With BRAF p.V600E, the limit of detection was 1% with 150 copies of the minor allele present in the analyzed DNA. With rs149041370, the detection limit was 0.1% with 15 copies of the minor allele present. Finally, the limit of detection of EGFR delE746-A750 was 0.02% with 3 copies of the minor allele present, approaching the stochastic limit of quantification. The estimated analytical specificity at these detection limits was >95%, based on a blinded analysis of 20 negative samples of each target (see Fig. 4 in the online Data Supplement).

**Discussion**

Minor mutant alleles can be enriched by snapback primer PCR. Wild-type strands experience intramolecular hybridization that inhibits primer extension, whereas mismatched mutant alleles do not block extension as effectively. Stated another way, the greater PCR efficiency of the mismatched allele relative to the matched wild-type allele selectively enriches for the mismatched allele. Enrichment is enhanced by rapid-cycle PCR with short extension times, a low Mg$^{2+}$ concentration, and a high $T_m$ of the snapback probe element.

Snapback primer analysis is 1 method of genotyping by duplex melting that does not use covalently labeled fluorescent probes. With the introduction of high-resolution melting analysis, such methods have
become popular and have recently been reviewed (29–31). These methods include small-amplicon genotyping (32), unlabeled-probe genotyping (26), and snapback primer genotyping (23). The characteristic feature of a snapback primer is a 5’ probe element that is complementary to its own extension product. After asymmetric PCR, the probe element hybridizes internally to its extension product and creates a hairpin loop. The hairpin is genotyped by melting in the presence of a PCR-compatible dye that detects heteroduplexes (23). When the probe element of the snapback primer and the template DNA are perfectly complementary, the stem of the

Fig. 4. The effect of extension time and Mg\(^{2+}\) concentration on allele enrichment with snapback primers.
Minor allele percentages were either 1% (○) or 0.1% (▲), and the extension temperature was 68 °C. (A), extension times varied between 0 s and 20 s at a free Mg\(^{2+}\) concentration of 1.2 mmol/L. (B), the free Mg\(^{2+}\) concentration was varied between 0.8 mmol/L and 2.2 mmol/L with an extension time of 0 s. The shorter the extension time and the lower the Mg\(^{2+}\) concentration, the higher the mutant allele fractions obtained. Experiments were performed in triplicate with a 5’-exonuclease–negative polymerase. Data are presented as the mean and SD.

Fig. 5. Analysis of thyroid nodules for BRAF p.V600E.
(A), Calibration curves for dual hybridization probes (HybProbes, ▲) and snapback primers (○), correlating the apparent mutation fraction to the actual mutation fraction. Triplicate reactions were analyzed; error bars indicate SDs. For snapback PCR, the extension temperature and time were 68 °C and 0 s, with 2 mmol/L Mg\(^{2+}\) and a wild-type probe T\(_m\) of 68 °C. Snapback primers overproduce the mutant product, as desired for an enrichment technique. (B), With the calibration curves in (A), the mutation fractions of 91 thyroid samples were measured by both HybProbe and snapback primer analysis (\(y = 1.1491x - 0.0228\); R\(^2\) = 0.9296). Results for needle aspirates (▲) and paraffin-embedded biopsies (○) are indicated.
Hairpin melts at a higher $T_m$ than for a mismatched template. When the template is heterozygous, there are 2 melting peaks, corresponding to the perfect match (wild type) and the mismatch (mutant).

Asymmetric heterozygous melting peaks observed during snapback genotyping provide both a challenge and an opportunity. The snapback probe element can inhibit the extension of its perfectly matched allele and thereby amplify the mismatched allele more efficiently, complicating genotyping; however, by characterizing and controlling this bias, the effect can be used to advantage for the enrichment of rare alleles.

The degree of allele enrichment is strongly related to the PCR’s extension time. When the extension time is 20 s (typical of conventional PCR) samples with a minor allele percentage of 0.1% do not enrich sufficiently for detection. As the extension time is reduced, however, these rare alleles become easy to detect. In the extreme case of an extension time of 0 s, the fraction of the mutant allele is increased from 0.1% to 29% (Fig. 4A). If the extension temperature is lower than the wild-type $T_m$, the wild-type product will snap into the hairpin configuration, blocking extension of wild-type strands. Mutant strands may still amplify, however, because the corresponding mismatched hairpins are less stable, but if the extension time is long enough (e.g., 20 s), the wild-type hairpin may be opened, permitting amplification to proceed at an efficiency nearly equal to that of the mutant allele. The improved sensitivity of short extension times has the added advantage of accelerating the PCR to completion in only 20–25 min (<20 s/cycle), even though 70 cycles are performed.

Rapid-cycle PCR has been around for almost 20 years (33, 34) and has recently been reviewed (35). Specificity advantages for allele-specific PCR have been noted (36), but under some conditions rapid cycling may limit PCR efficiency and introduce more variation into quantitative PCR (37). Even so, for allele enrichment the critical factor is the differential amplification efficiency of wild-type DNA compared with variant DNA, not the overall efficiency. The best enrichment occurs when the PCR efficiency is compromised, requiring more amplification cycles. Although 70 cycles is excessive by typical standards, cycling is complete within 25 min when rapid-cycle methods are used. Slower instruments are not expected to reach the enrichment obtained with the legacy carousel LightCycler instrument. Instead of amplifying on a carousel LightCycler and melting on the HR-1, another, more recent option is the LS-32 instrument (Idaho Technology), which combines the speed and capacity of the original LightCycler with the melting quality of the HR-1.

Low Mg$^{2+}$ concentrations limit strand reassocia-
tion (38). In our experiments, the free Mg$^{2+}$ concentration (i.e., Mg$^{2+}$ not chelated by dNTPs) ranged from 0.8 mmol/L to 2.2 mmol/L. Starting with 0.1% mutant alleles, snapback enrichment reached 48% as the Mg$^{2+}$ concentration was lowered (Fig. 4B). One explanation for this result is that a lower Mg$^{2+}$ concentration increases heterozygote amplification, thereby increasing the heteroduplex ratio (39). Another explanation is that the mutant allele does not form a hairpin at the lower Mg$^{2+}$ concentration, thus allowing the mutant allele to amplify more readily.

Snapback primer allele enrichment is a closed-
tube intramolecular method and requires no fluorescently labeled probes. Only 2 PCR primers are needed, the only addition being a tail of nucleotides on 1 of the primers. Minor alleles can be detected at relative concentrations down to 0.02%. Enrichment of minority alleles with snapback primers is rapid (<25 min), easy to design, inexpensive, and predictable. Conditions for snapback primer amplification can be modified for either genotyping (equal amplification of both alleles) or enrichment of 1 allele. Rare alleles of cancer-associated mutations can be detected in needle biopsies and in slides of formalin-fixed, paraffin-embedded tissue. Other settings in which this technique may be useful include prenatal testing and blood screening for early cancer detection. Snapback primer melting analysis provides a simple and effective tool for simultaneously enriching and detecting rare alleles.

Fig. 6. Snapback primer detection of EGFR exon 19 deletions in needle aspirates of lung masses.

The 6 thin black lines are melting curves from wild-type samples. The thicker line is a control sample of the EGFR deletion dele746–A750. The gray lines are from 2 clinical samples with the EGFR deletion delL747–P753insS. Snapback PCR was performed with an extension time and temperature of 0 s and 68 °C, a 2 mmol/L Mg$^{2+}$ concentration, and a wild-type probe $T_m$ of 78 °C. The hairpin $T_{hm}$ correlate with the length of the duplex stem for each allele.
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