Enriching Mutant Sequences by Modulating the Denaturation Time during PCR

To the Editor:

For minor and major DNA alleles, coamplification at lower denaturation temperature-PCR (COLD-PCR) selectively enriches minority alleles from mixtures of wild-type and mutation-containing sequences without a priori knowledge of the type or position of a mutation within a sequence (1). Since its inception (2), COLD-PCR has been employed in a variety of applications, including cancer, prenatal diagnostics, infectious diseases, and crop development (3). In COLD-PCR, mutation-containing sequences are enriched either because they have reduced melting temperatures (Tm) due to the type of mutations present (fast-COLD-PCR) or because they form heteroduplexes with wild-type DNA (full-COLD-PCR). In both cases the resulting Tm difference is exploited for preferential denaturation and replication of mutated sequences by employing a critical denaturation temperature (Tc) for a given DNA sequence. The Tc of an amplicon usually lies approximately 1 °C below the melting temperature (Tm) (4, 5). Because the critical denaturation temperature needs to be controlled precisely during PCR, a thermocycler with a high degree of accuracy and precision in temperature control is desirable, i.e., to within about ±0.2 °C. Unfortunately, this excludes several commonly used PCR machines. For example, the ECO real-time PCR machine from Illumina and the Lightcycler 1536 real-time PCR system from Roche are programmable to 1 °C increments, and the temperature of these instruments cannot be set to a finer degree. As such, these devices are unsuited for the current formats of COLD-PCR.

Approaches that can be used to circumvent this limitation are (a) amplicons can be designed with specific Tm values that instruments are capable of achieving to within ±0.2 °C and (b) amplicon Tm and the associated Tc can be altered with the addition of reagents, e.g., DMSO, that increase/decrease the Tm of the DNA duplexes to match denaturation temperatures that a PCR machine can achieve. These approaches are impractical because they require substantial optimization before COLD-PCR can be used.

To overcome the inherent limitations of some PCR instruments, we present an alternative approach, limited denaturation temperature–COLD-PCR (LDT-COLD-PCR). At a given denaturation temperature, the lower-melting, mutated amplicons are expected to denature faster than their wild-type counterparts, hence short denaturation times should favor mutant allele replication. LDT-COLD-PCR achieves mutation enrichment by limiting the duration of denaturation at temperatures equal to or above the Tm of the target amplicon.

To demonstrate the application of LDT-COLD-PCR, we selected a 146-bp v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) amplicon, with a Tm-decreasing p.G12V mutation, for which a Tc of 82.8 °C for 10 s on a finely regulated Cepheid machine demonstrated a 4–5-fold enrichment of mutations over wild-type DNA following PCR (data not shown). For this analysis, 161-bp amplicons were generated from 20 ng of wild-type and mutant genomic DNA with 20 cycles of PCR; samples were diluted 1:500 in DNase-RNase-free water before nested COLD-PCR.

When 83 °C or 82 °C denaturation for 10 s were set as the Tc on an ECO PCR machine, using a sample with a 10% ratio of mutant to wild-type genomic DNA, either no PCR product or no mutation enrichment were observed after PCR cycling. By setting the denaturation temperature to 85 °C, which lies approximately 1 °C above the Tm and running sequential COLD-PCR reactions at a range of denaturation times (8–20 s), we observed efficient PCR amplification. The mutation enrichment was then assessed by comparison to conventional PCR, performed in parallel. Sanger sequencing analysis of the products revealed progressive enrichment of the mutated target, with higher mutation enrichment associated with lower denaturation times at increased temperatures (Fig. 1). With a denaturation time of 8 s at 85 °C, the mutation abundance was enriched from an original 10% to 42%, similar to the enrichment observed on the Cepheid machine. Temperatures ranging from 84 °C to 89 °C were also examined (Fig. 1). Mutation enrichment was retained over a broad range of denaturation times and temperatures. Overall, the mutation enrichment diminished as the denaturation time lengthened.

LDT-COLD PCR analysis was performed using other KRAS amplicons harboring other Tm-reducing mutations such as p.G13D (G>A) and p.G12S (G>S). Analysis was also performed using a tumor protein p53 (TP53) exon 6 mutation. Similar results and levels of enrichment were observed for these tested amplicons (data not shown). We demonstrated that enrichment of mutations can be achieved...
by selecting temperatures up to 5 °C over the $T_m$ of an amplicon and shortening the denaturation time in PCR. The actual temperature attained by a sample is influenced by the lag between ramping the temperature and transferring heat to the sample. Accordingly, by modulating the denaturation time, the critical denaturation temperature necessary for mutation enrichment is achieved indirectly. Although COLD-PCR can be effective with instruments providing fine temperature control, LDT-COLD-PCR should be applicable with most PCR machines because denaturation times can be controlled to 1-s accuracy. Although our testing was limited to fast-COLD-PCR, we anticipate that the

Fig. 1. Enrichment of the KRAS p.G12V mutation with LDT-COLD-PCR assessed by Sanger sequencing analysis of the products (top). Estimates of mutant nucleotide abundance, relative to the wild-type nucleotide, were calculated from the peak height values of the chromatograms following triplicate experiments. Bottom, Summary of temperatures and times vs corresponding mutation abundance. SDs represent 3 independent experiments. The data depicted represent all points where sequenceable products were obtained and enrichment was observed.
current approach may also be applicable to other COLD-PCR formats, including full-COLD-PCR (6), ICE (improved and complete enrichment) COLD-PCR (7), and temperature-tolerant COLD-PCR (8).

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