Clinical Chemistry 58:7
1130–1138 (2012)

Temperature-Tolerant COLD-PCR Reduces Temperature Stringency and Enables Robust Mutation Enrichment

E. Castellanos-Rizaldos,[1] Pingfang Liu,[1] Coren A. Milbury,[1] Minakshi Guha,[1] Angela Brisci,[2] Laura Cremonesi,[2] Maurizio Ferrari,[2,3,4] Harvey Mamon,[5] and G. Mike Makrigiorgos[1,5*]

BACKGROUND: Low-level mutations in clinical tumor samples often reside below mutation detection limits, thus leading to false negatives that may impact clinical diagnosis and patient management. COLD-PCR (coamplification at lower denaturation temperature PCR) is a technology that magnifies unknown mutations during PCR, thus enabling downstream mutation detection. However, a practical difficulty in applying COLD-PCR has been the requirement for strict control of the denaturation temperature for a given sequence, to within ±0.3 °C. This requirement precludes simultaneous mutation enrichment in sequences of substantially different melting temperature (T_m) and limits the technique to a single sequence at a time. We present a temperature-tolerant (TT) approach (TT-COLD-PCR) that reduces this obstacle.

METHODS: We describe thermocycling programs featuring a gradual increase of the denaturation temperature during COLD-PCR. This approach enabled enrichment of mutations when the cycling achieves the appropriate critical denaturation temperature of each DNA amplicon that is being amplified. Validation was provided for *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) and *TP53* (tumor protein p53) exons 6–9 by use of dilutions of mutated DNA, clinical cancer samples, and plasma-circulating DNA.

RESULTS: A single thermocycling program with a denaturation-temperature window of 2.5–3.0 °C enriches mutations in all DNA amplicons simultaneously, despite their different T_ms. Mutation enrichments of 6–9-fold were obtained with TT-full-COLD-PCR. Higher mutation enrichments were obtained for the other 2 forms of COLD-PCR, fast-COLD-PCR, and ice-COLD-PCR.

CONCLUSIONS: Low-level mutations in diverse amplicons with different T_ms can be mutation enriched via TT-COLD-PCR provided that their T_ms fall within the denaturation-temperature window applied during amplification. This approach enables simultaneous enrichment of mutations in several amplicons and increases significantly the versatility of COLD-PCR.

In the area of personalized medicine for cancer, technologies that detect mutations and sequence DNA variants play an important role, because these alterations are related to therapeutic success, prognosis, and detection of residual cancer following surgical resection. The use of these technologies often presents challenges in the detection of minority alleles in clinical specimens, which can be masked by a high background of wild-type DNA. To date, there are different methodologies that address this issue and are capable of identifying known and/or unknown low-abundance mutations with high sensitivity *(1)*, including the recently described COLD-PCR (coamplification at lower denaturation temperature PCR) *(2–5)*. This novel form of PCR, developed by our group, enriches preferentially mutated DNA sequences over wild type by using a lower-temperature denaturation step during the cycling protocol [critical denaturation temperature (T_c)[6]]. The T_c is below the melting temperature (T_m) of the wild-type amplicon and permits the selective denaturation of the mutated sequences in every round of PCR, whereas wild-type sequences remain substantially double-stranded, thus amplifying less. Mutated DNA enrichment by COLD-PCR facilitates subsequent mutation detection. This method is applicable to

[1] Division of DNA Repair and Genome Stability, Department of Radiation Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA; [2] Genomic Unit for the Diagnosis of Human Pathologies, Center for Translational Genomics and Bioinformatics, San Raffaele Scientific Institute, Milan, Italy; [3] Università Vita-Salute San Raffaele, Milan, Italy; [4] Diagnostica e Ricerca San Raffaele SpA, Milan, Italy; [5] Department of Radiation Oncology, Dana-Farber Cancer Institute, Brigham and Women's Hospital, Harvard Medical School, Boston, MA.

* Address correspondence to this author at: Dana-Farber/Brigham and Women's Cancer Center, Brigham and Women's Hospital, Level L2, Radiation Therapy, 75 Francis St., Boston, MA 02115. Fax 617-582-6037; email mmakrigiorgos@lroc.harvard.edu.

Disclaimer: The contents of this manuscript do not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health.

Received January 27, 2012; accepted April 20, 2012.
Previously published online at DOI: 10.1373/clinchem.2012.183095

[6] Nonstandard abbreviations: T_c, critical denaturation temperature; T_m, melting temperature; HRM, high-resolution melting analysis; TT, temperature tolerant; LM-PCR, ligation-mediated PCR; T_{mmin}, minimum T_m.

both known and unknown mutation enrichment and can be integrated with downstream detection methodologies such as Sanger sequencing, high-resolution melting analysis (HRM), real-time PCR, and next-generation sequencing, among others *(6–9)*. COLD-PCR has been used successfully by diverse groups for mutation detection in different targets such as v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*)[7] *(2, 7, 10–15)*, *GNAS1* (GNAS complex locus) *(16)*; *IDH1* [isocitrate dehydrogenase 1 (NADP+)] *(17)*; *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) *(18)*; *EGFR* (epidermal growth factor receptor) *(11)*; fetal paternally inherited mutations of plasma *(19, 20)*; and peach floral genes *(21)*.

The main challenge in the application of COLD-PCR is to establish precisely the correct T_c, because a variation in critical temperature as small as ±0.2–0.3 °C could make it difficult to obtain satisfactory mutation enrichment. A consequence of this requirement is the inability to amplify diverse sequences simultaneously with a single thermocycling program because in general each amplicon has a different T_c. As such, it would be highly desirable to enable enrichment of mutations in multiple sequences in existing PCR thermocyclers or in emerging, high-throughput PCR platforms *(22, 23)* before amplicon sequencing.

In this report we present a temperature-tolerant (TT) COLD-PCR approach (TT-COLD-PCR) that relaxes the stringency on the denaturation temperature and simultaneously amplifies different targets with diverse T_c with a single cycling program. We combine TT-COLD-PCR with HRM *(24, 25)*, providing a rapid, simple, and sensitive screening method to confirm mutation enrichment before Sanger sequencing or other complimentary assays.

Materials and Methods

DNA AND TUMOR SAMPLES

Human male genomic DNA was obtained from Promega (cat. no. G1471) and used as a wild-type control. Genomic DNA from cell lines containing tumor protein p53 (*TP53*) mutations (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol58/issue7) was purchased from American Type Culture Collection. Cells from the A549 lung carcinoma cell line with a defined *KRAS* mutation (p.G12S, c.34G>A) were cultured in F12 medium supplemented with 10% fetal bovine serum at 37 °C with 5% CO_2. Genomic DNA from the A549 cells was extracted with a DNeasy Blood & Tissue Kit (Qiagen). DNA from clinical colorectal (n = 4), glioblastoma (n = 5), and lung (n = 1) cancer specimens, previously reported to have mutations at different abundances detected by use of COLD-PCR *(8, 26)* and other independent methods, were used to further validate the technology (see online Supplemental Table 2). We also tested plasma-circulating DNA (obtained from a radiation therapy patient who gave informed consent, with institutional review board approval) that demonstrated a *TP53* mutation. The plasma-circulating DNA was preamplified by ligation-mediated PCR (LM-PCR) as previously described *(27)*, along with plasma-circulating DNA from a healthy donor (see online Supplemental Table 2).

PREAMPLIFICATION WITH CONVENTIONAL PCR FROM GENOMIC DNA

We applied a conventional-PCR preamplification step from genomic DNA before testing TT approaches in a nested PCR format for the 3 forms of COLD-PCR, (fast-, full-, or ice-COLD-PCR). Primers for all forms of PCR were synthesized by Integrated DNA Technologies and are listed in online Supplemental Table 3. PCR was performed in a 25-μL final volume for all PCR reactions, with the Phusion™ high-fidelity DNA polymerase system (New England Biolabs).

Before performing nested TT-fast- and TT-ice-COLD-PCR, we conducted DNA preamplification by amplifying individual amplicons from genomic DNA to build enough template for subsequent COLD-PCR reactions. Before performing TT-full-COLD-PCR, we conducted genomic DNA preamplification of all *TP53* exons simultaneously using a single-tube multiplex-PCR approach as reported previously *(26)*, with minor modifications. Reactions were carried out in a 15-μL reaction volume consisting of 1× Phusion high-fidelity buffer, 0.2 mmol/L (each) dNTPs, 0.1 μmol/L (each) primers, and 0.6 U Phusion DNA polymerase. The resulting PCR product was then diluted 200-fold and used as a template for TT-full-COLD-PCR reactions, which were performed in separate tubes for each target amplicon. For comparison to COLD-PCR formats, a nested conventional PCR was always performed in parallel with TT-COLD-PCR.

CRITICAL DENATURATION TEMPERATURE WINDOW FOR TT-FULL-COLD-PCR

The T_c is the temperature(s) at which the majority of the major alleles are inhibited while the minor alleles are selectively amplified. Although in previous COLD-PCR reports a discrete temperature for a given amplicon was required for robust enrichment, we present here an alternative that eases the requirement for strin-

[7] Human genes: *KRAS*, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; *GNAS1*, GNAS complex locus; *IDH1*, isocitrate dehydrogenase 1 (NADP+); *BRAF*, v-raf murine sarcoma viral oncogene homolog B1; *EGFR* (epidermal growth factor receptor).

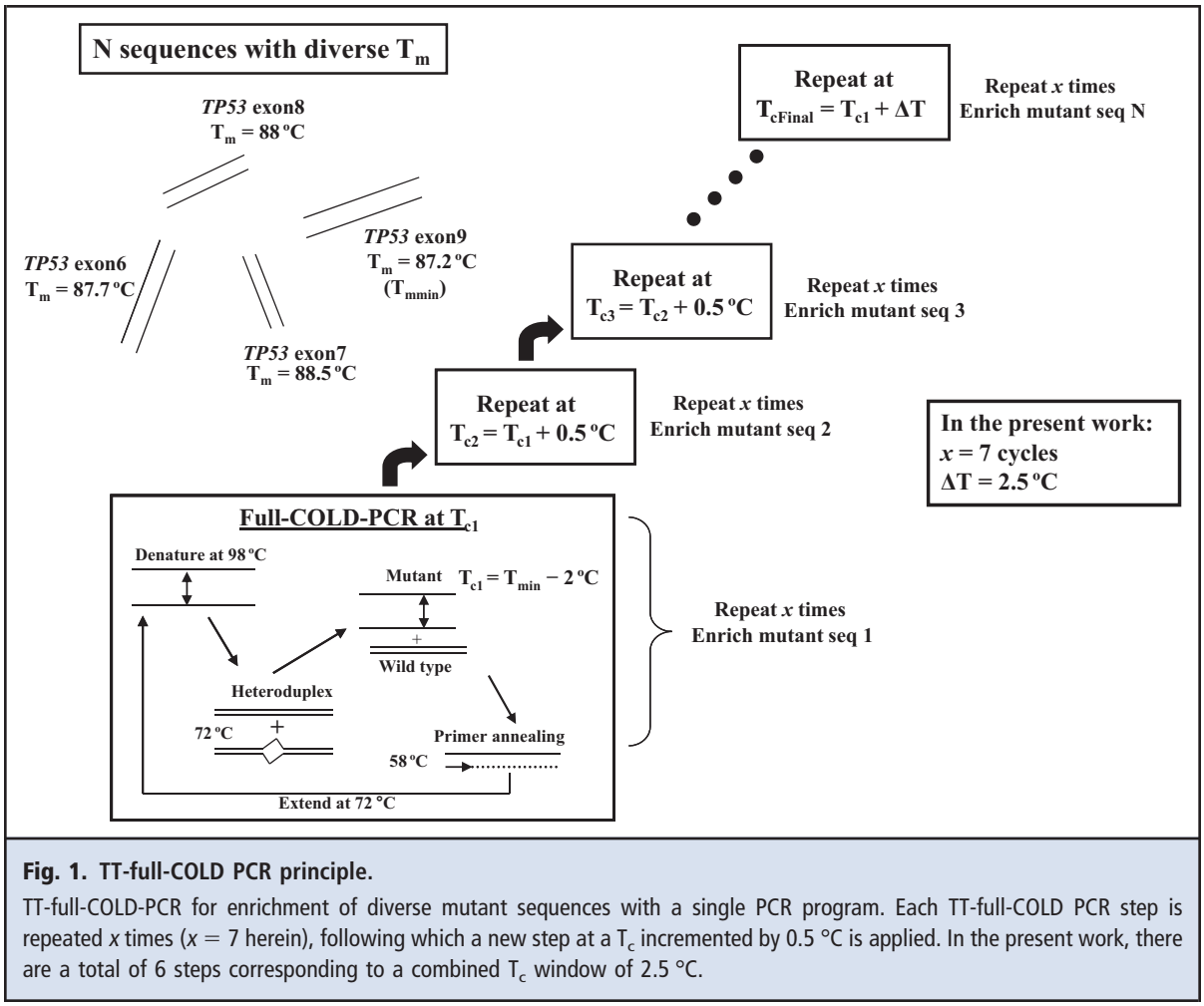

Fig. 1. TT-full-COLD PCR principle.

TT-full-COLD-PCR for enrichment of diverse mutant sequences with a single PCR program. Each TT-full-COLD PCR step is repeated *x* times (*x* = 7 herein), following which a new step at a T_c incremented by 0.5 °C is applied. In the present work, there are a total of 6 steps corresponding to a combined T_c window of 2.5 °C.

gency of the denaturation temperature while also offering satisfactory enrichment. The aim is to increase the denaturation temperature such that the mutant alleles are preferentially denatured and amplified before the wild-type alleles, even if they are present in amplicons with diverse T_c. To achieve this, T_ms of the wild-type target DNA sequences are experimentally determined with a conventional PCR in the presence of 1X LCGreen dye (Idaho Technologies), to define the initial T_c. Alternatively, the amplicon T_m can be predicted simply by use of bioinformatic tools *(28)*. The initial T_c applied for TT-full-COLD-PCR is ≥1 °C below the minimum T_m (T_{mmin}) among all target amplicons tested, so that when the temperature increases gradually it first encounters the T_c of the amplicon with the lowest T_m. Thus, for TT-full-COLD-PCR we adopted: initial $T_c = T_{mmin} - 2$ °C. The TT-full-COLD-PCR protocol applied here consists of successive steps, with 7 cycles each step, where the T_c is incremented at 0.5 °C intervals between successive steps (Fig. 1).

TT-FULL-COLD-PCR REACTIONS

PCR thermocycling conditions are described in online Supplemental Tables 4 and 6. Reaction conditions were the same for all cases except that for *KRAS* the concentration of primers was 0.075 μmol/L each.

TT-FAST-COLD-PCR REACTIONS

PCR products from the genomic DNA preamplification were diluted 500–1000-fold and used as a template for nested TT-fast-COLD-PCR reactions. We performed nested TT-fast-COLD-PCR on an Eppendorf Mastercycler machine (Eppendorf) using the "plate" block setting and "safe" temperature mode to ensure better well-to-well uniformity and to prevent overheating above the target temperatures (see online Supplemental Tables 4 and 5). A single TT-fast-COLD-PCR program was used to amplify *TP53* exons 6–9. For TT-fast-COLD-PCR we adopted: initial $T_c = T_{mmin} - 1.3$ °C (see online Supplemental Table 5). T_ms for exon 7 and 8 amplicons were effectively reduced by adding 4% and 2% DMSO, so that amplicon T_m fell within the

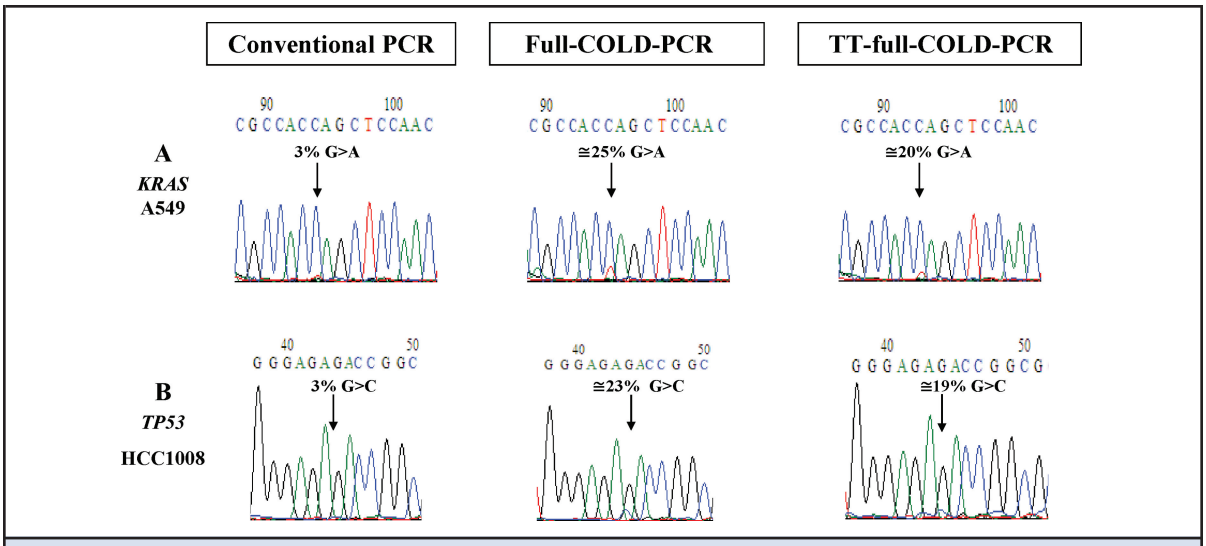

Fig. 2. TT-full-COLD PCR evaluation for a 3% mutation abundance following dilution of the *KRAS* codon 12 G>A mutation (A) and *TP53* exon 8 G>C mutation (B) into wild-type DNA.

TT-full-COLD PCR is compared to conventional PCR and to traditional full-COLD PCR performed at a single T_c and followed by Sanger sequencing.

TT-fast-COLD-PCR temperature window applied in this investigation.

TT-ICE-COLD-PCR REACTIONS

Amplification via ice-COLD-PCR inhibits preferentially the amplification of one DNA strand in wild-type DNA *(5)*, whereas the second strand remains uninhibited. In view of this effect, and to inhibit excessive amplification of the second strand, we performed the nested amplification reaction in an asymmetric-PCR mode, wherein the reverse primer was present at 5 times the concentration of the forward primer. Conventional PCR also was performed in asymmetric mode for comparative analysis. Reagent conditions for the conventional PCR were the same as reported for genomic amplification except for primer concentration, with 0.2 μmol/L forward primer and 1.0 μmol/L reverse primer, and 1 μL of the diluted (1:5000-fold) amplicon from the genomic DNA preamplification. Reagent conditions for the ice-COLD-PCR reactions were the same as in the conventional assays but with the additional incorporation of a reference sequence (RS) at a 25 nmol/L concentration. The RS used was the same as previously described *(5)*. For TT-ice-COLD-PCR, we examined a range of T_c values below the T_m of the amplicon tested before determining the optimal initial T_c of 83.7 °C that generated maximum enrichment for the temperature window applied (see online Supplemental Tables 7 and 8).

HRM AND SANGER SEQUENCING

We direcly analyzed TT-full-COLD-PCR and conventional amplicons using HRM on a Light Scanner HR96 system (Idaho Technologies) before sequencing. For Sanger sequencing, PCR products were digested by Exonuclease I/shrimp alkaline phosphatase and sequenced at Eton Bioscience. Owing to the short amplicon length, a 30-T tail was added to the 5′ end of the sequencing primer as reported previously *(29)*. Chromatograms were then analyzed with BioEdit v7.1.3 (Ibis Biosciences). Mutant allele abundances relative to wild type were estimated from the peak height values of the Sanger sequencing chromatograms as previously reported *(5)*. However, because Sanger sequencing is not quantitatively accurate, these estimations may not be precise.

Results

TT-FULL-COLD-PCR VS CONVENTIONAL PCR OR TRADITIONAL FULL-COLD-PCR

We evaluated the mutation enrichment obtained via TT-full-COLD-PCR compared to other forms of PCR. For this purpose, we amplified serial dilutions of DNA from mutation-containing cell lines for *KRAS* (c.34G>A mutation, p.G12S) and *TP53* exon 8 (c.841G>C mutation, p.D281H) by conventional PCR; TT-full-COLD-PCR; and traditional, single-T_c full-COLD-PCR before Sanger sequencing (Fig. 2). In both cases, a 3% mutant content was not visible in the chromatogram of the conventional amplification, but was visible and enriched to approximately

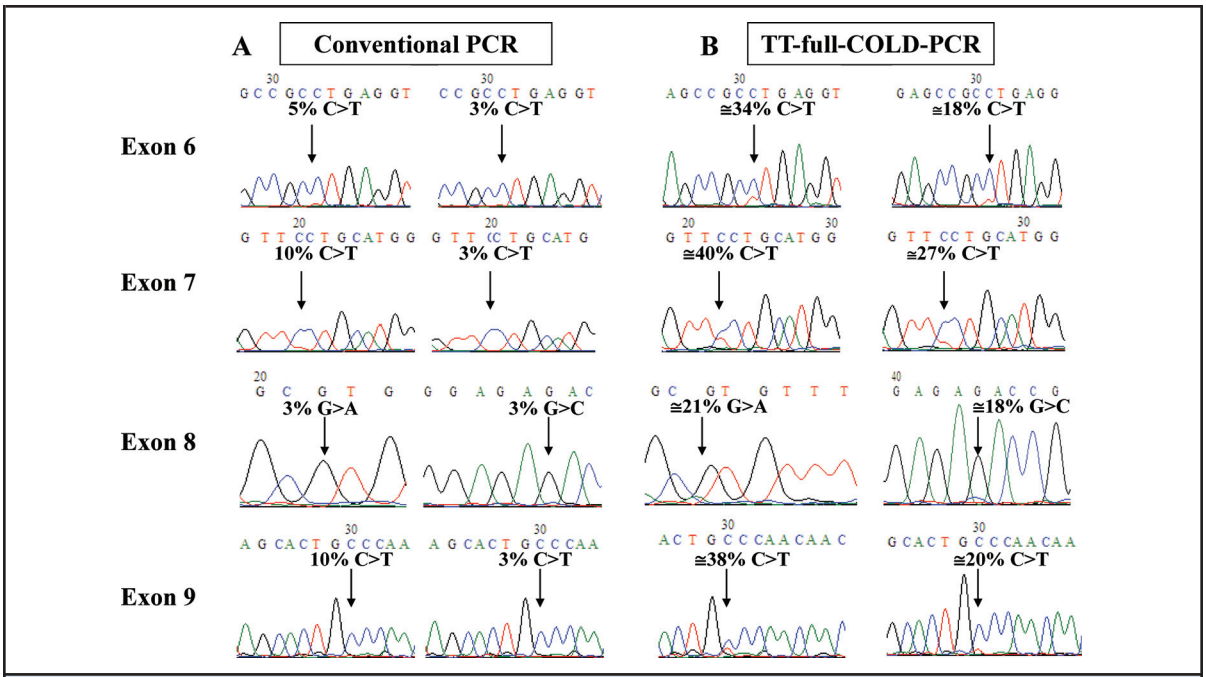

Fig. 3. Application of a single TT-full-COLD PCR thermocycling program to enrich mutations in exons 6–9 in *TP53*, using known serial dilutions into wild-type DNA.

Sanger sequencing chromatograms depict results from conventional PCR (A) and TT-full-COLD-PCR amplification (B). The left and right columns in frame A correspond to the left and right columns in frame B.

19%–25% mutant content for *KRAS* and *TP53* by traditional full-COLD-PCR and TT-full-COLD-PCR. The mutation enrichment, approximately 6–8-fold, was evident for both types of COLD-PCR. The enrichment obtained from TT-full-COLD-PCR was somewhat less than that of single-T_c COLD-PCR.

TT-FULL-COLD-PCR: A SINGLE THERMOCYCLING PROGRAM FOR DIVERSE *TP53* AMPLICONS

Using the scheme illustrated in Fig. 1, we applied universal thermocycling conditions spanning a window of denaturation temperatures from 85.2 to 87.7 °C to simultaneously amplify and enrich for mutations in 4 different regions of the *TP53* gene, exons 6–9. The protocol applied was capable of enriching mutation abundances by approximately 4–9-fold in all examined exons simultaneously, according to the results from Sanger sequencing (Fig. 3). TT-full-COLD-PCR amplicons were also examined by HRM before sequencing and compared to HRM from the conventional PCR protocol. Fig. 4 shows an example of 2 of the amplicons tested via HRM; 148 bp of *TP53* exon 6 (c.668C>T, T_m-reducing mutation) and 115 bp of *TP53* exon 8 (c.841G>C, T_m-equivalent mutation) are presented. The exon 6 mutation is evident via HRM but cannot be confirmed by sequencing, whereas the exon 8 mutation cannot be confirmed by either HRM or sequencing following conventional PCR. In contrast, following TT-full-COLD-PCR the 3% mutation abundance is detectable via both HRM and sequencing. Additional HRM testing of DNA from cell lines with T_m-reducing mutations in *TP53* exon 8 (c.818G>A) and exon 9 (c.925C>T) are presented in online Supplemental Fig. 1. On the basis of the magnitude of the HRM curve differentiation from wild-type DNA, we determined that the mutation detection sensitivity was notably higher in all TT-full-COLD-PCR amplicons compared to that obtained with conventional PCR amplicons. Finally, DNA from a cell line with a T_m-increasing mutation (PFSK-1, c.823T>G, p.C275G) for *TP53* exon 8 was diluted into wild-type DNA to a final 4% or 2% mutation abundance and tested with TT-full-COLD-PCR or conventional amplification strategies. The data indicate that a mutation enrichment of approximately 7–14-fold was obtained, on the basis of the sequencing chromatograms (see online Supplemental Fig. 2).

TT-FULL-COLD-PCR HRM TESTING OF CLINICAL SAMPLES WITH LOW-LEVEL MUTATIONS

To further evaluate TT-full-COLD-PCR, we screened DNA from previously tested lung, colorectal, and glioblastoma tumor samples *(26)* containing high-, mid-,

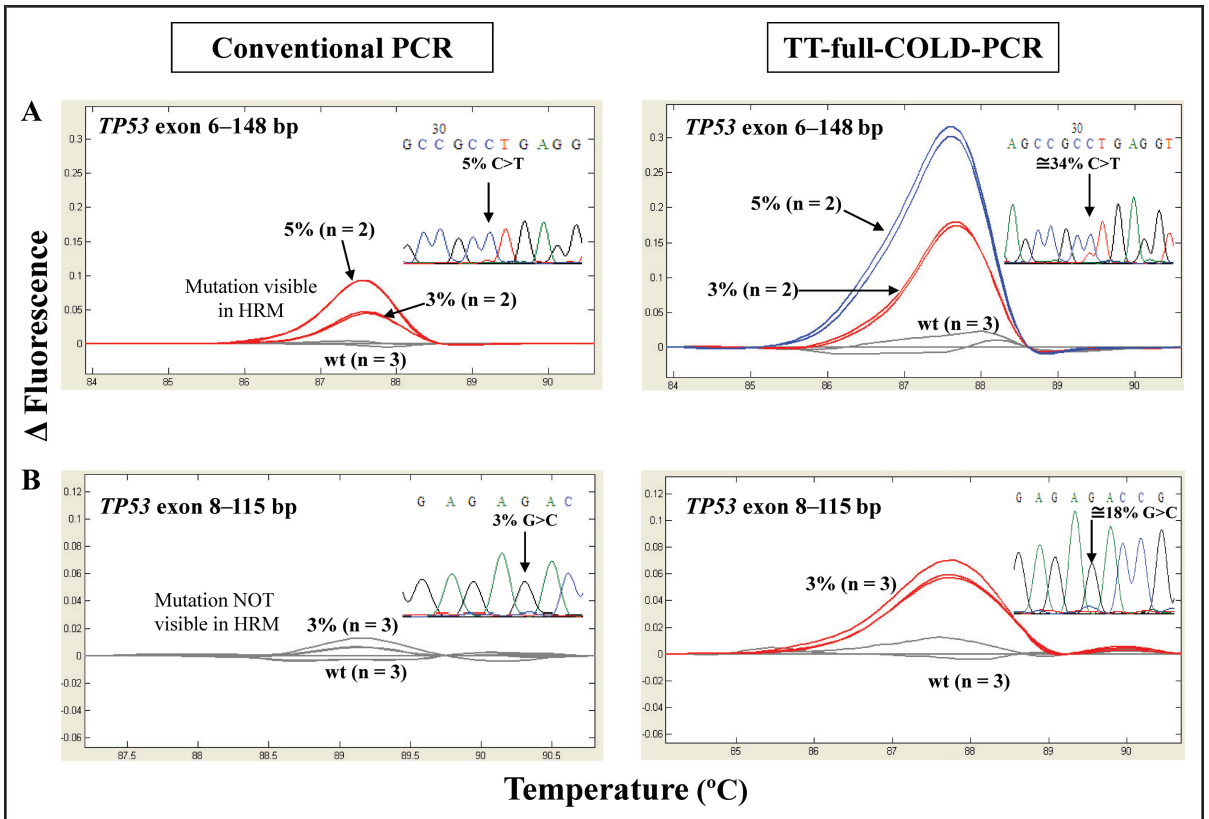

Fig. 4. HRM and sequencing after conventional PCR and TT-full-COLD PCR for *TP53* exon 6 (C>T mutation) and exon 8 (G>C mutation), with known serial dilutions into wild-type (wt) DNA.

Following conventional PCR, the 3% exon 6 mutation is detected via HRM but not via sequencing; the 3% exon 8 mutation is detected via neither method. Following TT-full-COLD these mutations are detected with both HRM and sequencing, although in exon8 the enrichment is marginally discriminated from background.

and low-abundance mutations (see online Supplemental Table 2). Clinical samples were amplified by TT-full-COLD-PCR and conventional PCR, evaluated by HRM, and validated by Sanger sequencing (Fig. 5; also see online Supplemental Fig. 3). Fig. 5 depicts HRM profiles from a clinical sample, CT20, containing low-level mutations in both *TP53* exons 8 and 9 following amplification with TT-full-COLD-PCR or alternatively via conventional PCR. Mutations are evident via HRM in both cases, but are visible only in the TT-full-COLD-PCR Sanger chromatograms, although the mutation in the exon 8 chromatogram is close to the limit of detection. Clinical samples containing mid- to high-abundance mutations were also examined by HRM and sequencing. These mutations were detectable via both conventional and TT-full-COLD-PCR (not shown).

TT-FAST-COLD-PCR: TESTING OF SERIAL DILUTIONS AND PLASMA-CIRCULATING DNA

Fast-COLD-PCR is useful for enriching the subset of mutations that decrease the T_m of the amplicons G:C>A:T and G:C>T:A, which comprise the majority of somatic mutations in human cancer *(7)*. Fast-COLD-PCR is a rapid protocol that generally results in higher enrichments than full-COLD-PCR. Thus, TT thermocycling was also adapted for fast-COLD-PCR format, for *KRAS* and *TP53* exons 6–9 mutations, in an Eppendorf thermocycler. Cycling conditions for *KRAS* spanned a temperature window from 82.7 to 85.3 °C, and for *TP53* a temperature window from 85.5 to 88.5 °C (see online Supplemental Tables 4 and 5).

We tested for *KRAS* mutation enrichment by diluting cells from the A549 cell line (mutant in c.34G>A, p.G12S) into wild-type DNA, amplifying by TT-fast-COLD-PCR or conventional PCR, and applying Sanger sequencing. Online Supplemental Fig. 4 demonstrates that after TT-fast-COLD-PCR, 3% and 10% mutation abundances increased to approximately 50% and 87%, respectively. Similarly, we applied a single TT-fast-COLD-PCR protocol for simultaneous enrichment of DNA with *TP53* mutations in exon 6 (SNU-182, p.S215I),

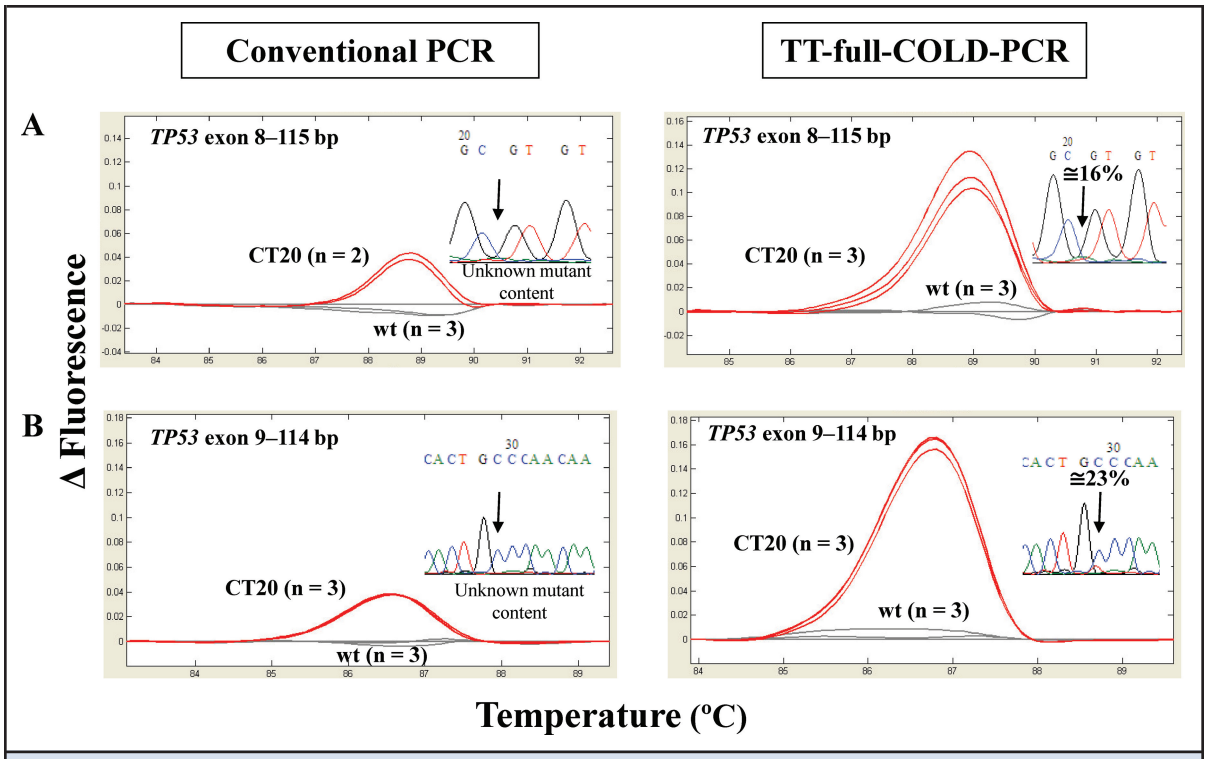

Fig. 5. Analysis of a colorectal cancer sample CT20 with low-abundance mutations that were detectable via HRM but not detectable via Sanger sequencing following conventional PCR.

The mutations in *TP53* exons 8 and 9 were detectable with both methods following TT-full-COLD.

exon 7 (HCC2157, p.R248W), exon 8 (SW480, p.R273H), and exon 9 (SW480, p.P309S). Subsequent Sanger sequencing revealed mutation enrichment in all exons (see online Supplemental Fig. 5). To also demonstrate the application of TT-fast-COLD-PCR in the enrichment of low-level mutations in a clinical sample, we tested plasma-circulating DNA from a radiation therapy patient in whom a low-level mutation in *TP53* exon 8 (c.847C>T, p.R823C) had been identified via traditional fast-COLD-PCR. Plasma-circulating DNA isolated from a normal volunteer was tested in parallel. We preamplified the pair of plasma-circulating DNAs was via LM-PCR using the method we described *(27)*. To exclude the possibility of a polymerase-introduced error during the LM-PCR preamplification step, we also validated the mutation using fast-COLD-PCR directly from unamplified DNA isolated from plasma (not shown). Sanger sequencing of the conventional PCR product revealed no mutation (Fig. 6). In contrast, TT-fast-COLD-PCR product revealed an enrichment of the mutation to an abundance of approximately 77% in the patient circulating DNA sample (Fig. 6).

TT-ICE-COLD-PCR

We also adapted the TT-COLD-PCR to ice-COLD-PCR and compared the mutation enrichment obtained via conventional PCR, TT-ice-COLD-PCR, and ice-COLD-PCR at a single T_c. We used DNA from a cell line with a T_m-equivalent mutation (HCC1008, p.841G>C, p.D281H) diluted into wild-type DNA to a final 3% mutation abundance. Following evaluation of a series of critical denaturation temperatures (see online Supplemental Table 8), it was shown that optimal mutation enrichment was observed when the initial T_c was set approximately 5 °C below the T_m of the amplicon:RS90 duplex, which was subsequently increased by 0.5 °C every 7 cycles, to a final T_c of 2.5 °C below the T_m of the amplicon:RS90 duplex. The Sanger-sequencing results demonstrated that both TT-ice-COLD-PCR and ice-COLD-PCR at a single T_c resulted in similar mutation enrichment, i.e., 3% mutation abundance was enriched to approximately 36% (see online Supplemental Fig. 6).

Discussion

COLD-PCR *(3, 4)* is inexpensive and relatively easy to implement because the primary required modification

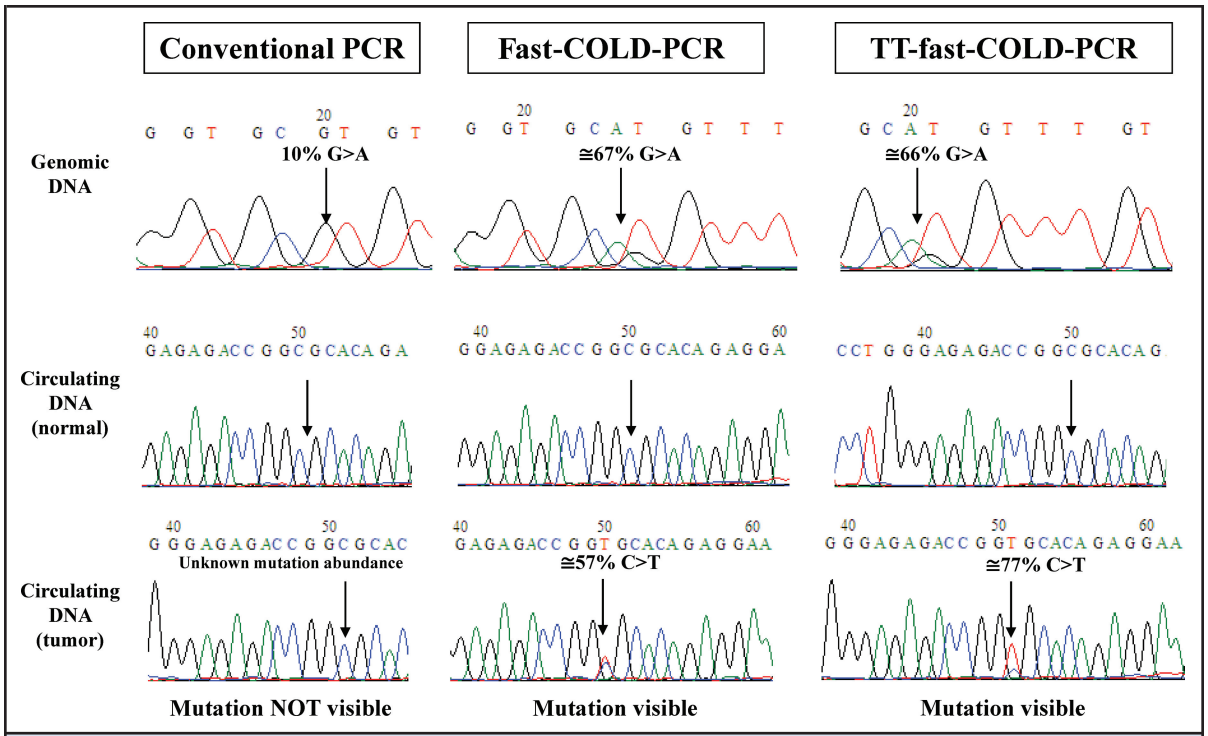

Fig. 6. TT-fast-COLD-PCR applied to circulating DNA.

Circulating tumor DNA isolated from plasma of a radiation therapy patient with a low-abundance mutation (c.847C>T, R283C) was analyzed by use of conventional PCR, fast-COLD PCR at a single T_c, and TT-fast-COLD-PCR. The mutational enrichment was compared to that obtained for a 10% mutation abundance and also with circulating DNA obtained from a healthy volunteer.

relative to conventional PCR lies in the cycling protocol and the denaturation temperature. However, the requirement for strict reproducibility and precision (±0.3 °C) in controlling the critical denaturation temperature can introduce significant variations between different laboratories, equipment, or experiments. Factors such as PCR buffer composition and lot may generate differences in amplicon T_m and T_c. Additionally, differences in temperature-ramping speed, or in actual temperature between wells in a single PCR machine, may also influence the mutation enrichment obtained during individual COLD-PCR reactions. To address such issues, in preliminary work Candau et al. *(30)* observed that gradual increase of denaturation temperature within a fine interval during fast-COLD-PCR ("touch-up" COLD-PCR) removes the well-to-well temperature inconsistencies that introduce variations to the mutation enrichment. This concept was expanded herein, to encompass a broader window of temperatures spanning 2.5–3 °C, to achieve simultaneous mutation enrichment in diverse amplicons of substantially different T_m by use of a single thermocycling program. TT conditions can be defined for all forms of COLD-PCR: full-, fast-, and ice-COLD-PCR. Using TT-full-COLD-PCR, we demonstrated simultaneous mutation enrichment in 4 *TP53* exons whose T_m spanned a 2.5 °C temperature window with the use of a single thermocycling program. It can thus be reasonably expected that mutations in any other amplicon whose T_m falls within the same temperature window would also be enriched, thereby enabling diverse PCR sequences to be enriched on a conventional PCR machine with a single thermocycling program in a high-throughput format. Furthermore, amplicons with a T_m exceeding the applied temperature window can also be included via amplification in the presence of DMSO or other modifiers that lower the T_m. It is possible to broaden the temperature window to encompass a wider range of T_m or to apply a finer step between temperatures, i.e., 0.3 °C instead of 0.5 °C. This change may boost further the capabilities of TT-COLD-PCR; however, caution is required to avoid primer dimers resulting from increasing PCR cycles. Additional considerations include polymerase inactivation resulting from repeated heating, or reaching the maximum number of PCR cycles allowed by the thermocycler software. In practice, a balance among all these factors should be accom-

plished to define optimal conditions for TT-COLD-PCR.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.

Research Funding: H. Mamon, NCI; G.M. Makrigiorgos, Innovative Molecular Analysis Technologies Program of the NCI, grants CA-111994 and CA-151164.
Expert Testimony: None declared.
Other: G.M. Makrigiorgos, Transgenomic royalties to Dana Farber Cancer Institute.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: A. Brisci participated in this study as partial fulfillment of her PhD in Molecular Medicine, Program in Predictive and Preventive Medicine, San Raffaele University, Milan, Italy.

Note: COLD-PCR is a technology that is owned by the Dana-Farber Cancer Institute and has been commercially licensed.

References

1. Milbury CA, Li J, Makrigiorgos GM. PCR-based methods for the enrichment of minority alleles and mutations. Clin Chem 2009;55:632–40.
2. Li J, Wang L, Mamon H, Kulke MH, Berbeco R, Makrigiorgos GM. Replacing PCR with COLD-PCR enriches variant DNA sequences and redefines the sensitivity of genetic testing. Nat Med 2008;14:579–84.
3. Li J, Milbury CA, Li C, Makrigiorgos GM. Two-round coamplification at lower denaturation temperature-PCR (COLD-PCR)-based sanger sequencing identifies a novel spectrum of low-level mutations in lung adenocarcinoma. Hum Mutat 2009;30:1583–90.
4. Li J, Makrigiorgos GM. COLD-PCR: a new platform for highly improved mutation detection in cancer and genetic testing. Biochem Soc Trans 2009;37:427–32.
5. Milbury CA, Li J, Makrigiorgos GM. Ice-COLD-PCR enables rapid amplification and robust enrichment for low-abundance unknown DNA mutations. Nucleic Acids Res 2011;39:e2.
6. Milbury CA, Li J, Liu P, Makrigiorgos GM. COLD-PCR: improving the sensitivity of molecular diagnostics assays. Expert Rev Mol Diagn 2011;11:159–69.
7. Song C, Milbury CA, Li J, Liu P, Zhao M, Makrigiorgos GM. Rapid and sensitive detection of KRAS mutation after fast-COLD-PCR enrichment and high-resolution melting analysis. Diagn Mol Pathol 2011;20:81–9.
8. Milbury CA, Correll M, Quackenbush J, Rubio R, Makrigiorgos GM. COLD-PCR enrichment of rare cancer mutations prior to targeted amplicon resequencing. Clin Chem 2012;58:580–9.
9. Li J, Wang L, Janne PA, Makrigiorgos GM. Coamplification at lower denaturation temperature-PCR increases mutation-detection selectivity of TaqMan-based real-time PCR. Clin Chem 2009;55:748–56.
10. Carotenuto P, Roma C, Cozzolino S, Fenizia F, Rachiglio AM, Tatangelo F, et al. Detection of KRAS mutations in colorectal cancer with Fast COLD-PCR. Int J Oncol 2012;40:378–84.
11. Santis G, Angell R, Nickless G, Quinn A, Herbert A, Cane P, et al. Screening for EGFR and KRAS mutations in endobronchial ultrasound derived transbronchial needle aspirates in non-small cell lung cancer using COLD-PCR. PLoS One 2011;6:e25191.
12. Pritchard CC, Akagi L, Reddy PL, Joseph L, Tait JF. COLD-PCR enhanced melting curve analysis improves diagnostic accuracy for KRAS mutations in colorectal carcinoma. BMC Clin Pathol 2010;10:6.
13. Kristensen LS, Daugaard IL, Christensen M, Hamilton-Dutoit S, Hager H, Hansen LL. Increased sensitivity of KRAS mutation detection by high-resolution melting analysis of COLD-PCR products. Hum Mutat 2010;31:1366–73.
14. Mancini I, Santucci C, Sestini R, Simi L, Pratesi N, Cianchi F, et al. The use of COLD-PCR and high-resolution melting analysis improves the limit of detection of KRAS and BRAF mutations in colorectal cancer. J Mol Diagn 2010;12:705–11.
15. Zuo Z, Chen SS, Chandra PK, Galbincea JM, Soape M, Doan S, et al. Application of COLD-PCR for improved detection of KRAS mutations in clinical samples. Mod Pathol 2009;22:1023–31.
16. Delaney D, Diss TC, Presneau N, Hing S, Berisha F, Idowu BD, et al. GNAS1 mutations occur more commonly than previously thought in intramuscular myxoma. Mod Pathol 2009;22:718–24.
17. Boisselier B, Marie Y, Labussiere M, Ciccarino P, Desestret V, Wang X, et al. COLD PCR HRM: a highly sensitive detection method for IDH1 mutations. Hum Mutat 2010;31:1360–5.
18. Pinzani P, Santucci C, Mancini I, Simi L, Salvianti F, Pratesi N, et al. BRAFV600E detection in melanoma is highly improved by COLD-PCR. Clin Chim Acta 2011;412:901–5.
19. Galbiati S, Brisci A, Lalatta F, Seia M, Makrigiorgos GM, Ferrari M, Cremonesi L. Full COLD-PCR protocol for noninvasive prenatal diagnosis of genetic diseases. Clin Chem 2011;57:136–8.
20. Du J, Zou X, Pan Y, Li SF, Lu GX. Non-invasive prenatal molecular detection of a fetal point mutation for congenital adrenal hyperplasia using co-amplification at lower denaturation temperature PCR. Chin Med J 2010;123:3343–6.
21. Chen Y, Wilde HD. Mutation scanning of peach floral genes. BMC Plant Biol 2011;11:96.
22. Chan M, Chan MW, Loh TW, Law HY, Yoon CS, Than SS, et al. Evaluation of nanofluidics technology for high-throughput SNP genotyping in a clinical setting. J Mol Diagn 2011;13:305–12.
23. Tewhey R, Warner JB, Nakano M, Libby B, Medkova M, David PH, et al. Microdroplet-based PCR enrichment for large-scale targeted sequencing. Nat Biotechnol 2009;27:1025–31.
24. Gundry CN, Vandersteen JG, Reed GH, Pryor RJ, Chen J, Wittwer CT. Amplicon melting analysis with labeled primers: a closed-tube method for differentiating homozygotes and heterozygotes. Clin Chem 2003;49:396–406.
25. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High-resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 2003;49:853–60.
26. Milbury CA, Chen CC, Mamon H, Liu P, Santagata S, Makrigiorgos GM. Multiplex amplification coupled with COLD-PCR and high resolution melting enables identification of low-abundance mutations in cancer samples with low DNA content. J Mol Diagn 2011;13:220–32.
27. Mamon H, Hader C, Li J, Wang L, Kulke M, Amicarelli G, et al. Preferential amplification of apoptotic DNA from plasma: potential for enhancing detection of minor DNA alterations in circulating DNA. Clinical Chemistry 2008;54:1582–4.
28. Dwight Z, Palais R, Wittwer CT. uMELT: prediction of high-resolution melting curves and dynamic melting profiles of PCR products in a rich web application. Bioinformatics 2011;27:1019–20.
29. Binladen J, Gilbert MT, Bollback JP, Panitz F, Bendixen C, Nielsen R, Willerslev E. The use of coded PCR primers enables high-throughput sequencing of multiple homolog amplification products by 454 parallel sequencing. PLoS One 2007;2:e197.
30. Candau R SH, Han Y, Eastlake P, Kaldjian E, Makrigiorgos GM, Gerard GF. Very high sensitivity detection of K-RAS Exon 2 mutations using fast COLD-PCR. Washington (DC):AACR; 2010.