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Combining COLD-PCR and High-Resolution Melting provides a highly sensitive approach for efficient scanning and identification of low-level unknown cancer mutations

HARVARD MEDICAL SCHOOL

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Background:

Molecular profiling of somatic mutations in cancer often requires the identification of low-level DNA mutations within an excess of wild-type DNA. However, the selectivity and sensitivity of the mutation detection methodology are often limiting in the reliable detection of low-prevalence mutations (Milbury *et al.* 2009). The recently developed COLD-PCR (CO-amplification at Lower Denaturation Temperature, Li *et al.* 2008) resolves several limitations in low-level mutation detection by utilizing critical denaturation temperatures to enrich mutation-containing amplicons during PCR irrespective of where the mutations are located on the sequence.

One major attribute of COLD-PCR is that mutant enrichment is typically sufficient for direct sequencing of low-prevalence mutations after PCR. Nevertheless, dideoxy-sequencing remains an expensive option, thus pre-screening sequences via a high-throughput scanning approach is attractive. High resolution melting (HRM) is a technique that can be used for pre-scanning for unknown mutations prior to sequencing but cannot specifically ascertain the identity of the detected mutation. Accordingly, to enable the enrichment, quick scanning, and identification of low-level unknown mutations, we combined COLD-PCR with HRM mutation scanning, followed by direct sequencing of mutation-positives.

Methods:

1. Serial dilutions of the cell lines HCC2157 and SW480 (ATCC) in genomic male DNA (Promega Corp.) were prepared in order to asses method sensitivity.
2. TP53 exons 7 and 8 were amplified via conventional-PCR and COLD-PCR. COLD-PCR was performed using a lower denaturation temperature (~1.0ºC below the amplicon melting temperature) to preferentially amplify mutation-containing DNA for any mutation along the sequence. A Cepheid SmartCycler II thermocycler was used.
3. PCR amplicons were subsequently screened for the presence of mutations via HRM on the LightScanner HR96 (Idaho Technologies Inc.).
4. Amplicons were sequenced at the Dana-Farber Molecular Biology core facility and the mutation position and identity was verified.
5. This approach was evaluated using DNA from human lung adenocarcinoma surgical samples.

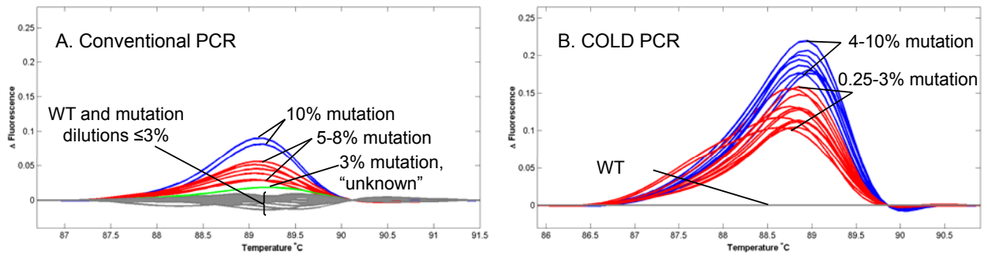

Figure 1. Representative HRM mutation detection sensitivity after conventional (A) and COLD (B) PCR amplification of the TP53 exon 8 amplicon; cell line SW480 was serially diluted in genomic DNA. Using COLD-PCR, sensitivity increased by 10 to 16-fold.

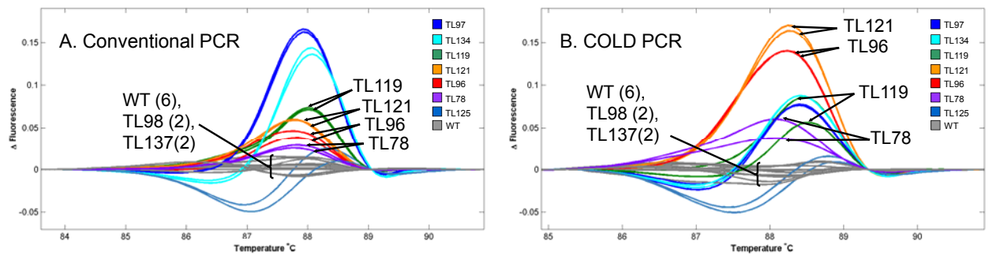

Figure 2. HRM lung tumor mutation detection sensitivity after conventional (A) and COLD (B) PCR of the TP53 exon 7 amplicon.

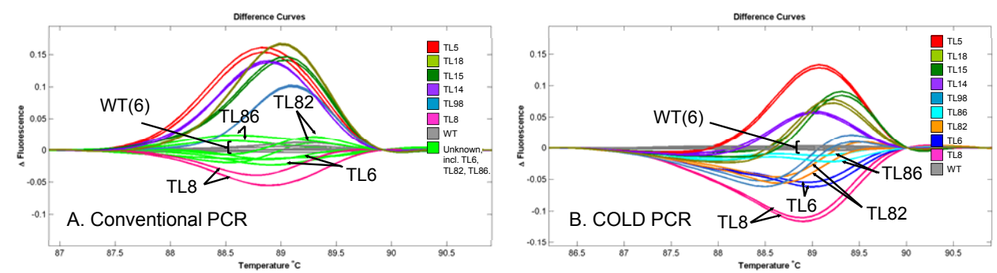

Figure 3. HRM lung tumor mutation detection sensitivity after conventional (A) and COLD (B) PCR of the TP53 exon 8 amplicon.

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2. Li, J., Wang, L., Mamon, H., Kulke, M.H., Berbeco, R. and Makrigiorgos, G.M.: Replacing PCR with COLD-PCR enriches variant DNA sequences and redefines the sensitivity of genetic testing. Nature Medicine 14 (2008) 579-584.

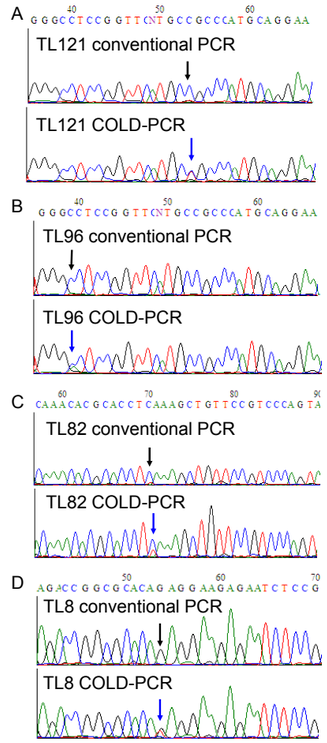

Figure 4. Representative sequence chromatograms of conventional and COLD-PCR amplicons for TP53 exon 7 (A & B) and exon 8 (C & D).

Results:

Serial Dilutions for HRM Sensitivity:

- HRM analysis of conventional PCR amplicons exhibited a mutation detection limit of 4.0% (**Figure 1A**) to 10.0% (exon 7).
- HRM analysis of COLD-PCR amplicons exhibited a mutant detection limit of 0.25% (**Figure 1B**) to 1.0% (exon 7).
- This demonstrates a 10-20-fold improvement via COLD-PCR enrichment and HRM detection over conventional PCR-HRM.
- TP53 exon 7 mutation enrichment is typically less efficient (~10-fold increase) than that of TP53 exon 8 (~16-fold increase) due to the presence of two melting domains (data not shown).

Lung Tumor HRM Analysis:

- In TP53 exon 7, both conventional PCR-HRM and COLD-PCR-HRM detected all seven previously identified mutations (**Figure 2**); two were included as WT controls.
- In TP53 exon 8, conventional PCR-HRM reliably detected six low-level mutations, however three were scored as 'unknown', indicating the possible presence of a mutation.
- HRM analysis of the COLD-PCR amplicons confirmed all nine low-level mutations (**Figure 3**), including the three classified as 'unknown' via conventional PCR-HRM.

Sequence Analysis:

- Of the 16 mutations evaluated here, half (8 of 16) could not be identified reliably by sequencing conventional PCR amplicons.
- Sequence analysis of the COLD-PCR amplicons correctly identified 94% of the mutations in exon 7 and 100% of the mutations in exon 8 amplicons. Representative sequence chromatograms are presented in **Figure 4**.
- Despite mutation enrichment via COLD-PCR, one mutation (sample TL78) could not be identified via sequencing and may present the lower limit of mutation detection for the TP53 exon 7 amplicon. We speculate that the TL78 mutation exists at <1% among wild-type alleles.

Conclusions:

1. COLD-PCR increases the detection capability of HRM and subsequently enables identification of the position and type of low-level mutations via sequencing of mutation-positive samples. This facilitates greatly evaluation of the potential clinical significance of low-level mutations.
2. The power of this approach is also apparent in the direct sequence analysis of COLD-PCR amplicons. Low-level mutations that are well below the sensitivity level of standard sequencing (i.e. <20%) can be identified easily after COLD-PCR enrichment.
3. Using this improved approach we demonstrate that COLD-PCR-HRM has the potential to be used as a routine diagnostic screening tool that combines speed, ease of use, low-cost, sensitivity, and ability to identify the position and type of unknown low-level mutations.