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COLD-HRM PCR versus Conventional HRM PCR to Detect the *BRAF* V600E Mutation

A Real Improvement?

To the Editor-in-Chief:

In the recent article by Mancini et al,[1] the authors demonstrated the improved sensitivity of the detection of *KRAS* mutations and the *BRAF* V600E mutation using coamplification at lower temperature PCR (COLD-PCR) in combination with high-resolution melting (HRM) instead of conventional PCR followed by HRM. They reported an eightfold higher sensitivity for *KRAS* mutations (from 6.2% to 0.8%) and a debatable fourfold higher sensitivity for the *BRAF* V600E mutation (from 12.5% to 6.2%, which would seem to be a twofold higher sensitivity), using serial dilutions of DNA from cell lines harboring the mutations (CCRF-CEM and SKMel28) and DNA from MCF-7 as wild-type control.

Using conventional PCR and HRM, we[2] and others, including the authors,[3] have previously demonstrated detection limits for the *BRAF* V600E mutation of less than 5% mutated DNA in a background of wild-type DNA, which is more sensitive than the COLD-PCR assay described in their present article. In addition, our system[2] proved to be applicable to FFPE material. With FFPE samples, the spread of the melting curves increases due to the lower quality of DNA; however, we were able to distinguish samples containing 5% mutated DNA in a background of wild-type DNA.

We read with great interest the publications on the advantages of COLD-PCR. Indeed, we have used an extensive optimization process to try to improve the sensitivity of the *BRAF* assay using COLD-PCR instead of conventional PCR. We designed new primer sets that yield short amplicons to maximize the temperature effect caused by the mismatch in the heteroduplexes during COLD-PCR, taking into account single-nucleotide polymorphisms and the pseudogene described for the *BRAF* gene. We optimized the reaction composition and the PCR protocol. Unfortunately, we have as yet not been able to improve the sensitivity of 5% mutated DNA (SK-Mel28) in wild-type DNA (human mononuclear cell DNA) by using COLD-PCR, and we could not observe the expected shift in the quantification cycle values caused by the preferential amplification of the minority alleles (unpublished data). We could, however, confirm the equal melting behavior of 100% homozygously mutated SK-Mel28 DNA and 100% wild-type DNA in the difference plots (unpublished data). Thus, with regard to Figure 1 (right) in the study by Mancini et al,[1] we do not understand the different melting behavior of curves A (MCF-7 wild-type DNA) and G (100% SKMel28) because no heteroduplexes can be formed in these reactions and the T-A substitution should not cause any alteration in the melting behavior (in consideration of the hydrogen bonds).

Thus, although COLD-PCR has been described as a method ideal for the detection of low-level mutations by increasing the sensitivity of PCR-based assays, the application of COLD-PCR for the detection of the *BRAF* V600E mutation could not improve the sensitivity of our assay (unpublished data). Similar results have been shown by Fadhil and colleagues.[4] In addition, the intensive optimization and increased time necessary for each run (approximately 6 hours) renders COLD-PCR a sophisticated but labor-intensive method.

Although the optimization of COLD-PCR is still being pursued in our laboratory, the findings thus far are rather modest. We request that the authors comment on (1) the different melting behavior of the MCF-7 wild-type DNA and the SKMel28 DNA in the difference plots and (2) the failure to refer to other studies,[2] including their own work,[3] showing a detection limit of 5% mutated *BRAF* alleles using HRM in combination with conventional PCR.

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Author's Reply:

The use of coamplification at lower temperature PCR (COLD-PCR) has been demonstrated to be a valuable technique to enrich the percentage of mutated alleles in complex samples, such as DNA purified from cancer biopsy specimens[5] or maternal plasma.[6] In particular, the advantage of COLD-PCR to improve *KRAS* mutation detection in colorectal cancer has been recently demonstrated.[7] The efficiency of mutation enrichment is estimated in the range of fivefold to eightfold for melting temperature-retaining mutations and threefold to fivefold for melting temperature-increasing mutations.

It is important to recall that all of the experiments to calculate the sensitivity are based on a theoretical assumption, deriving from observations in dilutions of DNA from two cell lines harboring a wild-type and mutated genotype, respectively. This type of experimental procedure can be useful in the set-up of the method, but the essential goal is to increase the clinical sensitivity, that is, to increase clinical samples clearly positive for a somatic mutation.

Concerning the thermal shift between the two reference DNAs mentioned by Stadelmeyer and colleagues above, we must recall that T>A (A>T) substitutions when homozygous induce a small variation of melting temperature (approximately 0.2°C) and therefore also a possible shift in the HRM profile. This feature is not due to the number of hydrogen bonds but to the difference in the sequence (eg, adjacent bases and internal homology). This phenomenon could be influenced by the choice of primer sets or buffer composition. In any case, in our type of study, based only on the detection of somatic mutations with a variable and unpredictable level of heterozygosity, the thermal shift between homozygous sequences does not appear relevant.

Going back to the apparent differences between our experience and that of Stadelmeyer et al, it is important to recall that the use of different primers can generate different results. We have observed that different sets of primers for the same sequence can provide a very different sensitivity.

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