COLD-PCR Finds Hot Application in Mutation Analysis

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With the rapid development of targeted therapy and personalized medicine, gaining knowledge of the genetic and molecular characteristics of a patient’s tumor has become a crucial step in therapeutic and prognostic decision-making in oncology. A good example is the administration of therapy targeting the epidermal growth factor receptor (EGFR)2 in cancer patients. Several studies have shown that mutations that cause constitutive activation of the KRAS3 oncogene (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) predict resistance to anti-EGFR therapy (1–3), whereas activating mutations in EGFR predict response to therapy and favorable clinical outcome (4, 5). Because of such findings, the clinical demand for mutation testing has exploded recently in cancer diagnostics.

The specimens used for molecular testing range from large surgical resections to tiny fine-needle aspiration biopsies to cytology smears. Samples are submitted as either frozen or formalin-fixed, paraffin-embedded tissues, and the percentage of tumor cells varies substantially among different specimens. One of the major challenges that molecular diagnostics laboratories face is to detect mutations in samples with a low percentage of mutation-carrying tumor cells in a background of wild-type nonmalignant cells. The fact that the mutation-detection limit is approximately 20% for the gold standard of Sanger sequencing and approximately 5%–10% for pyrosequencing (6) necessitates implementation of tumor-enrichment strategies for samples with a small tumor component.

When tissue sections or smears on slides are used, tumor enrichment can be achieved at 2 levels: microscopically via manual microdissection or laser-capture microdissection and submicroscopically at the DNA level. Use of microdissection, one of the commonly used methods for tumor cell enrichment, requires substantial resources and expertise and prolongs the turnaround time. Alternatively, several sensitive methods have been developed to enrich specific mutations at the DNA level. These latter approaches can bring the detection limit down to 1%. Examples of such methods include the PCR-based shifted termination assay (Mutector®; TrimGen Corporation) (7) and the non-PCR–based Invader assay (Third Wave Technologies/Hologic) (8); however, these assays require post-PCR manipulations and can be expensive. Methods such as denaturing HPLC and high-resolution melting curve (HRM) analysis enable detection of mutations, including unknown ones, at levels as low as 0.1% (9, 10). These low-level mutations, however, are not amenable to downstream sequencing analysis.

COLD-PCR (coamplification at lower denaturation temperature–PCR) (11) is a recently developed modification of the PCR that has shown promise for meeting the limitations we currently face with detecting mutations in tumor samples. This method takes advantage of the dramatic drop in PCR amplification that occurs for perfectly matched homoduplex templates when the denaturation temperature is less than the critical denaturation temperature (Tc). By running the PCR at a reduced denaturation temperature, heteroduplexes of the mutant and wild-type sequences are selectively amplified, thus enriching the original minority mutant sequences to concentrations close to those of the majority sequences in the mixture. This method is very cost-effective in terms of equipment and reagents and is relatively easy to implement. Studies have shown that COLD-PCR can substantially increase mutation-detection sensitivity by 5- to 100-fold with different downstream detection methods, such as direct sequencing and restriction enzyme digestion, in various sample types (12–14).

In this issue of Clinical Chemistry, Milbury and coworkers describe yet another application of COLD-PCR in mutation detection by combining it with HRM analysis. HRM is an easy, fast, and low-cost method for screening variant sequences that has become widely implemented in molecular-testing laboratories (15). The authors demonstrate the applicability of COLD-PCR for HRM analysis by comparing conventional PCR/HRM with COLD-PCR/HRM for the detection of mutations in TP53 (tumor protein p53) exons 6–8 in human lung adenocarcinoma samples. By serially di-

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2 Nonstandard abbreviations: EGFR, epidermal growth factor receptor; HRM, high-resolution melting curve; COLD-PCR, coamplification at lower denaturation temperature–PCR; Tc, critical denaturation temperature.

3 Human genes: KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; TP53, tumor protein p53.
luting DNA extracted from a TP53 mutation-bearing cell line into DNA carrying the wild-type sequence, the authors have shown that COLD-PCR/HRM enabled an approximately 6- to 20-fold increase in detection sensitivity over conventional PCR/HRM analysis, pushing the detection limit from approximately 2%-10% mutant down to approximately 0.1%-1% mutant in mixtures with wild-type DNA. More importantly, because COLD-PCR selectively enriches the mutant allele in the amplification product, almost all (94%) of the mutations from fresh frozen lung tumor samples that were detected by COLD-PCR/HRM were successfully sequenced, compared with only 50% with conventional PCR.

The applicability of this approach in mutation detection, however, requires further validation. Unfortunately, the authors did not provide in their report the tumor percentage for the samples tested except for mentioning that the tumors were manually microdissected. The utility of COLD-PCR will be greatly enhanced if this approach can be demonstrated to completely eliminate microdissection steps or at least to substantially reduce the tumor percentage required in clinical samples for sequencing analysis. Additionally, considering that samples submitted for mutation testing today are mostly formalin-fixed, paraffin-embedded tissues, it will be highly useful if future studies can demonstrate similar results with formalin-fixed samples. Although COLD-PCR is very promising, technical hurdles still need to be addressed for this technique to be broadly adopted. Mostly, determining the $T_c$ for a particular sequence can be tricky. There is no standard algorithm for selecting the optimal $T_c$ other than following the general rule of thumb of typically about 1 °C below the experimentally determined amplicon melting temperature. Because of the nature of this method, amplicons with multiple melting domains and mutations that retain or increase the melting temperature will not be enriched. Therefore, before application of this method, a careful design of the amplicon and PCR conditions is crucial for successful detection of low-level mutations.

Finally, as with any other available highly sensitive mutation-detection method, selecting the right method for an application is important. Although high mutation-detection sensitivity is clearly desired when following patients for minimal residue disease, the correlation between a low-level of a mutation in newly diagnosed tumors and the response to therapy is still not known. For instance, the necessity of detecting a KRAS mutation in a small fraction of tumor cells (<1%) and thereby removing the patient from the potential benefit of the therapy is still debatable.

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