

Chapter 2

COLD-PCR: Applications and Advantages

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Abstract

Co-amplification at lower denaturation temperature-based polymerase chain reaction (COLD-PCR) is a single-step amplification method that results in the enhancement of both known and unknown minority alleles during PCR, irrespective of mutation type and position. This method is based on exploitation of the critical temperature, T_c , at which mutation-containing DNA is preferentially melted over wild type. COLD-PCR can be a good strategy for mutation detection in specimens with high nonneoplastic cell content, small specimens in which neoplastic cells are difficult to micro-dissect and therefore enrich, and whenever a mutation is suspected to be present but is undetectable using conventional PCR and sequencing methods. We describe in this chapter our COLD-PCR-based pyrosequencing method for *KRAS* mutation detection in various clinical samples using DNA extracted from either fresh or fixed paraffin-embedded tissue specimens.

Key words Polymerase chain reaction (PCR), Co-amplification at lower denaturation temperature PCR (COLD-PCR), *KRAS*, Mutation detection

1 Introduction

PCR works like a molecular photocopying machine. It makes millions of identical, amplified copies of a specific segment of DNA for downstream analysis. PCR is a key tool for DNA fingerprinting, forensic analysis, prenatal testing for genetic diseases, and finding mutations in cancer cells [1, 2].

PCR plays a vital role in detection of mutations in oncology specimens, most commonly when variant DNA sequences exist in the presence of a large majority of wild-type alleles as in case of heterogeneous tumors [3]. PCR does not contain an inherent selectivity towards variant (mutant) allele; thus both variant and non-variant alleles are amplified with approximately equal efficiency. The burden of identifying and sequencing a mutation in a PCR product falls on downstream assays. Despite being reliable for screening germline or somatic mutations, sequencing of unknown low-prevalence mutations using this otherwise powerful technology is still problematic.

With the rapid development of targeted therapies 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. 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 [4].

One of the major challenges that clinical molecular diagnostics laboratories face is detection of mutations in samples with a low percentage of mutation-carrying tumor cells in the background of nonmalignant cells. The fact that the mutation detection limits of conventional sequencing techniques such as the gold standard Sanger sequencing is approximately 20 % and that of pyrosequencing is approximately 5–10 % suggests that we need introduction of tumor enrichment strategies for samples with a small tumor component [3, 5].

When tissue sections or smears on slides are used, tumor enrichment can be achieved at two 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. Several sensitive methods have been developed to enrich specific mutations at the DNA level and bring detection limit down to 1 %. Examples of such methods include the PCR-based shifted termination assay and the non-PCR-based Invader assay [6, 7]; 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 %. These low-level mutations, however, are not amenable to downstream conventional sequencing analysis [8, 9].

COLD-PCR is a new form of PCR that preferentially enriches “minority alleles” from mixtures of wild-type and mutation-containing sequences, irrespective of where a known or unknown mutation is present in the DNA sequence that is being interrogated [10–12]. COLD-PCR is 10–100 times more sensitive, depending on the specific DNA involved, than standard PCR for detecting genetic changes. This method utilizes a critical denaturation temperature to enrich unknown mutations at any position on the sequence, during amplification. The critical denaturation temperature (T_c) is lower than standard denaturation temperatures, and the method is usually applied for amplicons less than ~200 bp in length; hence in some cases more than one amplicon must be used to cover the full target region [11].

COLD-PCR can be used in any of the two formats, *full* COLD-PCR or *fast* COLD-PCR, depending on whether it is important to

identify all possible mutations or achieve the highest mutation enrichment [10]. *Full* COLD-PCR enriches all possible mutations along the sequence, where an intermediate hybridization step temperature is used during PCR cycling to allow cross-hybridization of mutant and wild-type alleles (heteroduplexes). In *fast* COLD-PCR formation of heteroduplexes is not required and mutations are enriched anywhere along the sequence. The characteristic of both formats of COLD-PCR is to maintain adequate mutation enrichment for downstream sequencing, thus allowing the identification of the exact nucleotide change of low-prevalence mutations, though *fast* COLD-PCR has usually a greater mutation enrichment [12].

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 [13].

In summary COLDPCR can be a good strategy for mutation detection in specimens with high nonneoplastic cell content, in small specimens in which neoplastic cells are difficult to microdissect and therefore enrich, and whenever a mutation is suspected to be present but is undetectable using conventional PCR and sequencing methods.

2 Materials

- Fresh or formalin-fixed and paraffin-embedded (FFPE) tissue samples.
- Genomic DNA extraction system, such as Autopure LS automated DNA extraction system (Qiagen, Valencia, CA) for fresh blood and bone marrow samples, or Qiagen QIAamp DNA FFPE Tissue Kit for FFPE samples.
- Oligonucleotide primers.
- AmpliTaq Gold polymerase (Life Technologies).
- PCR master mix:
 - Forward and reverse primers.
 - Deoxynucleotide triphosphate (dNTP) mix.
 - MgCl_2 .
 - 20 mM Tris-HCl (pH 8.4).
 - 50 mM KCl.
- Thermal cycler, such as Applied Biosystems® 2720 Thermocycler (Life Technologies).
- Agarose gel equipment.
- Pyrosequencer (Qiagen) and reagents for downstream sequencing.

3 Methods

3.1 Overview of the General Principle

The principle of COLD-PCR is based on the observation that there is a critical denaturation temperature (T_c) for each DNA sequence that is lower than its melting temperature (T_m). PCR amplification efficiency for a DNA sequence drops abruptly if the denaturation temperature is set below its T_c . Even a single-nucleotide mismatch anywhere along the DNA sequence will generate a small but predictable change to the T_m . Depending on the sequence context and position of the mismatch, T_m can change 0.2–1.5 °C for a sequence of 200 bp. T_c is strongly dependent on DNA sequence, and it is determined empirically for each specific sequence. Figure 1 demonstrates the T_m profiles of the wild-type and mutant sequences at each of the six nucleotides of codons 12–13 of the *KRAS* gene. Studies have shown that PCR reaction yields no detectable amplification product when PCR denaturation temperature is set to below the T_c . The COD-PCR method takes advantage of this characteristic of PCR amplification to selectively enrich the minority alleles differing by one or more nucleotides at any position of a given sequence. In COLD-PCR, an intermediate annealing temperature is used during PCR cycling to allow cross-hybridization of mutant and wild-type alleles; heteroduplexes, which melt at lower temperatures than homo-duplexes, are then selectively denatured and amplified at T_c , while homo-duplexes remain double stranded and do not amplify efficiently. By fixing the denaturation temperature at T_c , mutations at any position along the sequence are enriched during COLD-PCR amplification.

3.2 COLD-PCR for *KRAS* Mutation Detection

Here we use *KRAS* mutation detection as an example to demonstrate the design of a COLD-PCR assay for clinical applications. A key consideration in designing a COLD-PCR assay that selectively amplifies the minority mutant alleles is to determine a new reduced denaturation temperature for the reaction. Ideally, this reduced denaturation temperature allows mainly the heteroduplexes to be denatured and amplified, and leaves the homo-duplexes double stranded and not amplified efficiently. We used the same primers as used in our conventional PCR assay, which produce an amplicon of 98 bp with 41.8 % of GC content and a T_m of 70.9 °C. Using the Poland algorithm, we plotted T_m profiles of the wild-type sequence along with those of mismatched sequences at each base pair within



Fig. 1 Nucleotide sequence of the 98 bp PCR product of the *KRAS* gene and T_m profiles of the wild-type and mutant sequences at each of the 6 nucleotides of codons 12–13. Arrows indicate primer sequences (*underlined*). Sequence of codons 12–13 is indicated in *red*. Figure legends indicate T_m profiles for wild-type (“wild”) and mutant (“m” and nucleotide number, e.g., “m25”) sequences

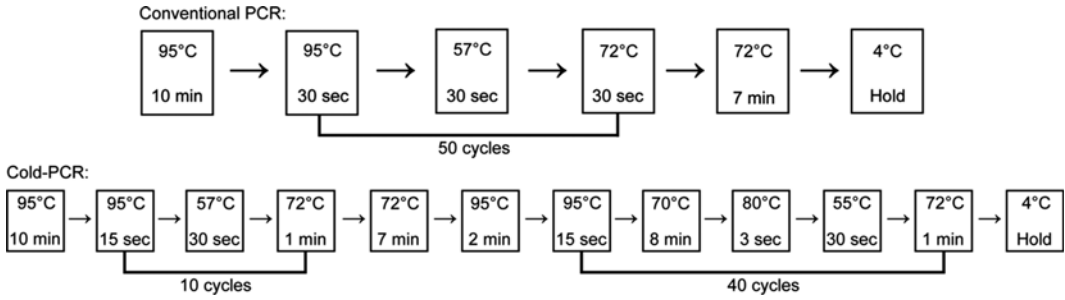


Fig. 2 Description of the PCR reaction conditions used in this study for conventional PCR and COLD-PCR protocols

KRAS codons 12 and 13 (Fig. 1). Based on this information, we set the reduced denaturation temperature of the COLD-PCR reaction at 80 °C. The reaction protocol started with 10 cycles of conventional PCR amplification for an initial buildup of all amplicons, followed by 40 COLD-PCR cycles to selectively enrich for mutant sequences. The initial conventional PCR cycling conditions are summarized as follows: 95 °C for 10 min; 10 cycles at 95 °C for 15 s, 57 °C for 30 s, and 72 °C, for 1 min; 72 °C for 7 min; and 95 °C for 2 min. Then 40 cycles of COLD-PCR were performed at 95 °C for 15 s, 70 °C for 8 min, 80 °C for 3 s, 55 °C for 30 s, and 72 °C for 1 min (Fig. 2). Full COLD-PCR reactions were performed on an ABI 2720 Thermocycler (Applied Biosystems). The COLD-PCR products were electrophoresed in agarose gels to confirm successful amplification of the 98 bp PCR product prior to pyrosequencing. As with our routine sequencing assay, a positive control, a negative control, and a reagent control were included in each run. All samples were run in duplicate.

3.3 Determining the Sensitivity of a COLD-PCR Assay

Sensitivity determination is a critical aspect of clinical assay development. A serial dilution study is usually performed for this purpose. For the *KRAS* COLD-PCR assay described above, we used a patient DNA sample containing a GGT-to-GCT mutation at codon 12 of the *KRAS* gene as the source of the mutant allele. This mutation-containing DNA sample was serially diluted with a wild-type DNA sample to obtain 1:2, 1:4, 1:8, 1:16, 1:32, 1:40, and 1:50 mutant to wild-type mixtures. All of the DNA mixtures were simultaneously subjected to conventional PCR and COLD-PCR followed by pyrosequencing. All samples were run in duplicate. The mutant-to-wild-type ratio was defined as the ratio of the peak height of a single mutant nucleotide over the peak height of a single wild-type nucleotide on pyrograms.

Figure 3 shows the pyrograms from the dilution study. With conventional PCR amplification, the mutant nucleotide peak on pyrogram became indistinguishable at 1:8 dilution, while it was clearly present at 1:32 dilution with COLD-PCR. Because the

mutant sample we used for the dilution study was heterozygous at *KRAS* codon 12, a 1:32 dilution translates to about a 1.5 % of detection sensitivity, which is four times better than the 6 % sensitivity obtained with conventional PCR method.

As shown in Fig. 4, with increasing serial dilutions, the mutation-to-wild-type ratio decreased proportionally in a

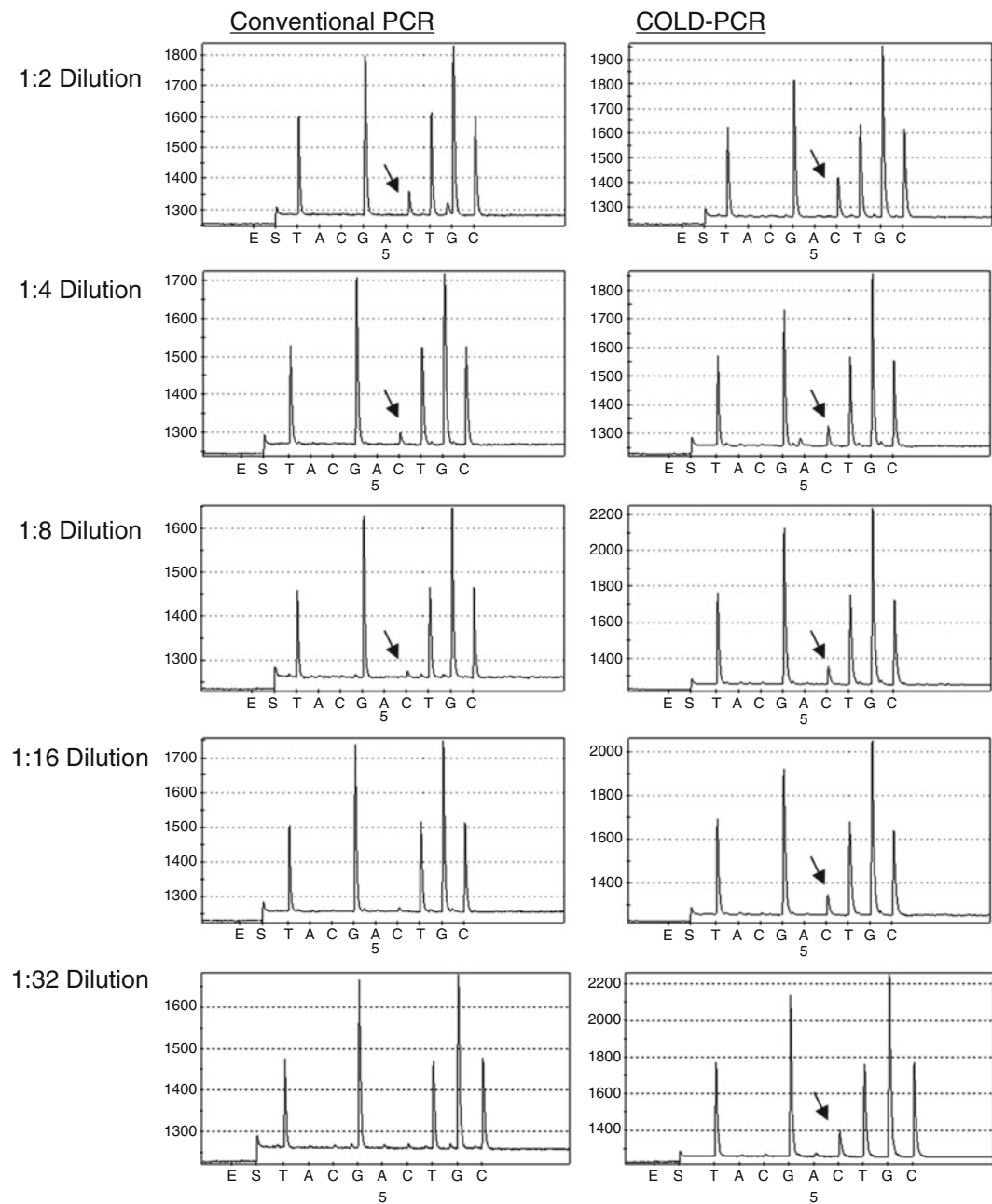


Fig. 3 Sensitivity determined by dilution study. Representative pyrograms of serial dilutions study with conventional PCR and COLD-PCR

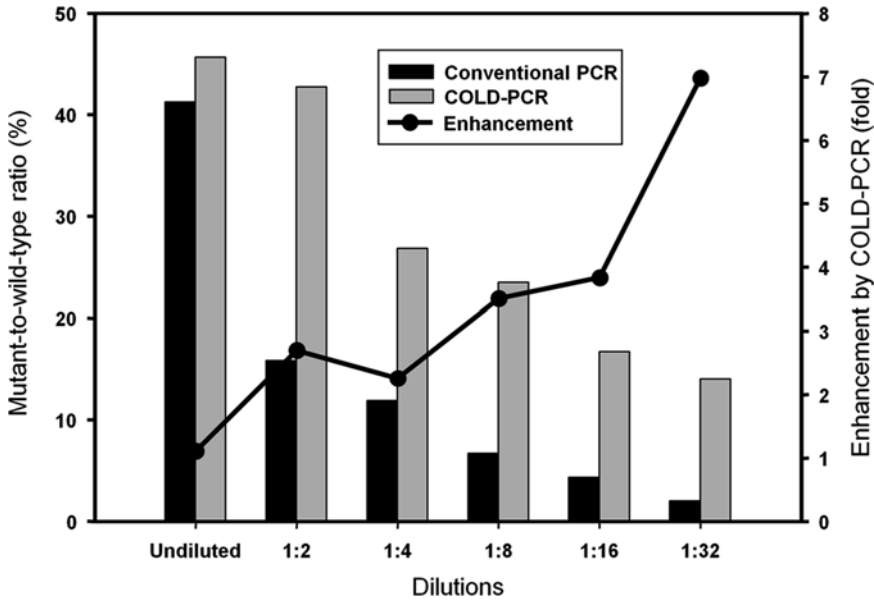


Fig. 4 Comparison of pyrosequencing results from undiluted to 1:32 dilution after conventional PCR and COLD-PCR (bars), and enhancement of COLD-PCR in serial dilutions (line)

first-order kinetics manner in both conventional PCR and COLD-PCR. The enhancement of mutation detection by COLD-PCR increased exponentially with increasing dilution. With the original specimen, which contains approximately 50 % mutant allele, COLD-PCR and conventional PCR had roughly the same efficiency in amplifying mutant alleles. At 1:32 dilution, COLD-PCR showed a sixfold greater efficiency in amplifying mutant allele compared with conventional PCR.

Within the detectable dilution range, all duplicate analyses of COLD-PCR products produced consistent pyrosequencing results. Different runs from the same sample also generated very similar results (correlation coefficient, 0.923).

4 Notes

1. COLD-PCR provides a general platform to improve the sensitivity of essentially all DNA variation detection technologies, such as Sanger sequencing, pyrosequencing, quantitative PCR, mutation scanning, genotyping, and methylation assays. As a general rule, a substantial enrichment for most COLD-PCR reactions can be obtained by using a T_c approximately 1 °C lower than the amplicon T_m ; for certain sequences, however, fine-tuning of the T_c can be beneficial, and an optimal T_c can vary 0.5–1.5 °C lower than the T_m . The T_m can be experimentally determined on most real-time thermocyclers by performing a melting curve after PCR.

2. COLD-PCR can be applied in two formats, *full* COLD-PCR and *fast* COLD-PCR, depending on whether it is important to identify all possible mutations or to achieve the highest mutation enrichments. The example that we have demonstrated above is *full* COLD-PCR format. The *fast* COLD-PCR is a rapid PCR setup that skips the intermediate cross-hybridization step at 70 °C. For mutation enrichment to occur, the *full* COLD-PCR protocol requires the buildup of substantial PCR product to achieve efficient cross-hybridization, which restricts the enrichment to the late stages of PCR. In contrast, for *fast* COLD-PCR there is no requirement for PCR product buildup; hence the mutation enrichment starts at earlier PCR cycles than for *full* COLD-PCR. *Fast* COLD-PCR is rapid and results in higher enrichments than *full* COLD-PCR. However, in order to enrich for all possible mutations, including deletions/insertions, the *full* COLD-PCR program is necessary. By applying the *full* COLD-PCR program, a mismatch will always form between mutant and wild-type sequences and enrichment occurs irrespective of whether the specific nucleotide change tends to increase or decrease the T_m .
3. COLD-PCR selectivity for point mutations can increase further if subsequent PCR rounds are performed. As with deep sequencing approaches that use single-molecule sequencing, COLD-PCR enrichment of mutations is ultimately limited by polymerase-introduced errors. As newer polymerases with very high fidelity are continuously being improved, however, so are the ultimate enrichment abilities of approaches like COLD-PCR. Ultradeep sequencing following several rounds of COLDPCR could reveal aspects of cancer biology that are clinically very important (e.g., the origins of resistance to therapy).
4. Theoretical limitations of COLD-PCR: as amplicon size increases the difference between T_m and T_c decreases; amplification of polymerase-induced errors (preferentially use proof-reader); requires optimization for each specific amplicon; well-to-well deviation from programmed temperature in many thermocyclers.

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