Chapter 33

COLD-PCR Enriches Low-Level Variant DNA Sequences and Increases the Sensitivity of Genetic Testing

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Abstract

Detection of low-level mutations is important for cancer biomarker and therapy targets discovery, but reliable detection remains a technical challenge. The newly developed method of CO-amplification at Lower Denaturation temperature PCR (COLD-PCR) helps to circumvent this issue. This PCR-based technology preferentially enriches minor known or unknown variants present in samples with a high background of wild type DNA which often hampers the accurate identification of these minority alleles. This is a simple process that consists of lowering the temperature at the denaturation step during the PCR-cycling protocol (critical denaturation temperature, T_c) and inducing DNA heteroduplexing during an intermediate step. COLD-PCR in its simplest forms does not need additional reagents or specific instrumentation and thus, can easily replace conventional PCR and at the same time improve the mutation detection sensitivity limit of downstream technologies. COLD-PCR can be applied in two basic formats: fast-COLD-PCR that can enrich T_m-reducing mutations and full-COLD-PCR that can enrich all mutations, though it requires an intermediate cross-hybridization step that lengthens the thermocycling program. An improved version of full-COLD-PCR (*i*mproved and *c*omplete *e*nrichment, ice-COLD-PCR) has also been described. Finally, most recently, we developed yet another form of COLD-PCR, temperature-tolerant-COLD-PCR, which gradually increases the denaturation temperature during the COLD-PCR reaction, enriching diverse targets using a single cycling program. This report describes practical considerations for application of fast-, full-, ice-, and temperature-tolerant-COLD-PCR for enrichment of mutations prior to downstream screening.

Key words Coamplification at lower denaturation temperature (COLD-PCR), Mutation detection, Low-level mutations, Cancer, Diagnosis, Prognosis, Therapy targets

1 Introduction

In the era of personalized medicine, the detection of rare DNA variants in biological specimens is important as it may influence certain clinical decisions in the fields of cancer, prenatal diagnosis, or infectious diseases [1–5]. As commonly applied, PCR does not contain an inherent selectivity towards variant (mutant) alleles, thus both variant and wild type alleles are amplified with similar

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efficiencies according to their original concentrations. The burden of identifying a low-level mutation falls on downstream assays, such as Sanger sequencing, pyrosequencing, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), restriction fragment length polymorphism (RFLP), denaturing high-performance liquid chromatography (dHPLC), and others.

We recently developed CO-amplification at Lower Denaturation temperature (COLD-PCR) [6], a new form of PCR that preferentially amplifies rare variants in the presence of a high background of wild type DNA, regardless of mutation type or position in the target of interest. The use of COLD-PCR during the amplification step from genomic DNA increases the sensitivity of detection of these low-level mutations prior to using downstream detection technologies such as Sanger sequencing, pyrosequencing, next-generation sequencing, mutation scanning, and mutation genotyping [7].

COLD-PCR enriches low-level mutations within the region of interest by reducing the denaturation temperature during PCR. There are several assays currently available that have the ability to enrich mutations during the process of PCR. For example, peptide nucleic acid (PNA)-based assays [8] can inhibit wild type DNA amplification by forming a PCR clamp or by preventing the primer from annealing to the wild type allele, thus preferentially amplifying the mutated allele. Alternatively, the Fluorescent Amplicon Generation (FLAG) assay [9] employs a highly thermostable restriction enzyme *Psp*GI in the PCR reaction that continuously digests the wild type allele leaving the mutant allele to be amplified during the course of PCR. In each of these two examples, and several others, the disadvantage is that they are designed to enrich only known mutations and therefore low-level unknown mutations remain undetectable [10]. COLD-PCR uniquely enables enrichment of unknown low-level mutations, irrespective of their type or position on the amplicon.

The principle of COLD-PCR is illustrated in Fig. 1. A single-base alteration anywhere along the sequence results in a small variation of the melting temperature of the amplicon (T_m) between 0.2 and 1.5 °C in 200 bp length amplicons [11, 12]. The temperature below the T_m has been defined as *critical denaturation temperature* (T_c). Amplicons differing by a single nucleotide result in variable PCR amplification efficiencies when the PCR denaturation temperature is set to the T_c. This important observation can be used during PCR for the selective amplification of minority alleles differing by one or more nucleotides at any position of a given sequence.

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 degree of mutation enrichment. The detailed methodological approach of full- and fast-COLD-PCR is presented in Fig. 1a, b, respectively.

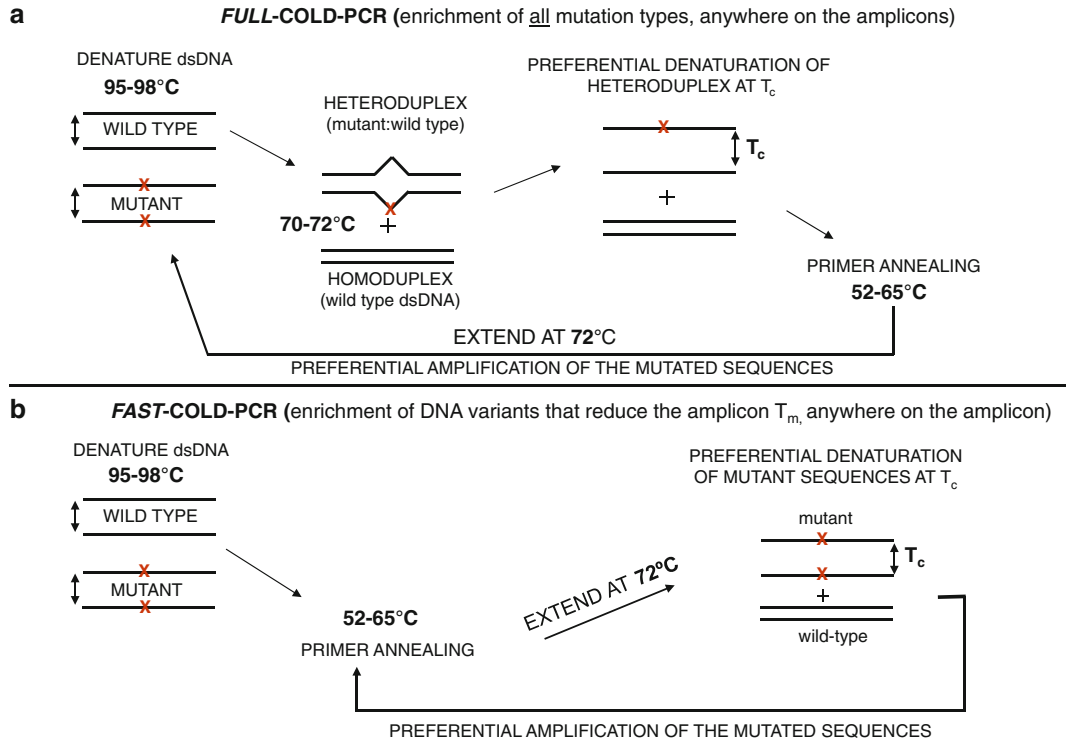

Fig. 1 *COLD-PCR protocol schematic* [6]. The two main forms of COLD-PCR reported full-COLD-PCR (*panel* **a**) and fast-COLD-PCR (*panel* **b**) are described. (**a**) *Full-COLD-PCR*: after denaturation (95.0–98.0 °C), single strands of DNA are re-annealed and cross-hybridized at 70.0–72.0 °C. Cross-hybridization of mutated DNA strand with the wild type DNA strand forms a mismatch-containing structure (heteroduplex), which is more unstable and denatures at a lower melting temperature than a fully matched structure (wild type: wild type, homoduplex). The next step in PCR cycle is to go to the Critical Denaturation Temperature (T_c) to denature most of these heteroduplexed structures. Then primer annealing and extension occur, which favors the amplification of mutated alleles. (**b**) *Fast-COLD-PCR* is simpler and faster to use than full-COLD-PCR, but is only applicable to mutations that decrease the melting temperature of the mutant amplicon compared to the wild type amplicon. Instead of the standard denaturation temperature, the critical temperature (T_c) preferentially denatures mutated alleles, followed by primer annealing and extension. This process preferentially allows the amplification of the T_m decreasing mutated alleles

In full-COLD-PCR (Fig. 1a), an intermediate hybridization step (70.0–72.0 °C) is used during the PCR cycling to allow heteroduplex formation (cross-hybridization of mutant and wild type alleles). These DNA heteroduplexes are less stable than homoduplexes resulting in lower denaturation temperatures. Therefore, when the denaturation temperature is set at the T_c, the majority of heteroduplex molecules denature and amplify over subsequent PCR cycles, whereas most homoduplex molecules remain double-stranded, reducing the amplification efficiency of the most abundant allele (wild type). By using the T_c instead of the regular denaturation temperature (95.0–98.0 °C) throughout the course of PCR, DNA variants at any position in the interrogated amplicon

are enriched during COLD-PCR amplification. In fast-COLD-PCR (Fig. 1b) the heteroduplex formation step is not necessary and setting the denaturation temperature at the T_c will favor the amplification of homoduplex molecules that contain T_m-reducing mutants (G:C>A:T or G:C>T:A). The process of full-COLD-PCR is somewhat longer than conventional PCR due to the incorporation of a cross-hybridization step to enable formation of heteroduplexes that may result in modest enrichment levels. The enrichment obtained by full-COLD-PCR is generally less than the one obtained with fast-COLD-PCR, because the formation of heteroduplexes is only efficient over the last 10–15 cycles prior to the reaction reaching saturation. To improve on this aspect, a new form of COLD-PCR was developed, ice-COLD-PCR, that provides improved and complete enrichment of all mutation types [13]. Briefly, ice-COLD-PCR incorporates a single-strand, wild type-specific oligonucleotide (reference sequence, added in excess within the reaction), which enables formation of heteroduplexes even in early PCR cycles. This reference sequence contains a 3′ modification (phosphate group) that helps to prevent polymerase extension during PCR amplification, and is somewhat shorter on both sides relative to the PCR amplicon so that primers do not bind to it. The thermocycling program is similar to that of *full-COLD-PCR*; an excess of wild type oligonucleotides incorporated in the reaction binds to other wild type alleles in the sample and at the T_c the mutant allele heteroduplex preferentially denatures and amplifies during PCR, while the wild type allele amplification is inhibited. However, it is important to notice that for all of these COLD-PCR forms a strict control of the T_c is required to attain optimal enrichment. More recently, we reported on a novel form of COLD-PCR, temperature-tolerant (TT) COLD-PCR that circumvents the T_c stringency and allows the amplification of different targets applying one set of cycling conditions [14]. This method includes a gradual increase of the T_c, a step-up protocol that spans a 1.5–3.0 °C window in small temperature increments (e.g., 0.3 °C) favoring the enrichment of mutations whose denaturation temperature is gradually reached during the reaction. This protocol has been adapted for every form of COLD-PCR, fast-, full-, and ice-COLD-PCR, allowing all mutation types to be enriched during the cycling protocol.

2 Materials

2.1 COLD-PCR Reagents and Equipment

1. Wild type genomic DNA samples, such as reference human male genomic DNA (Promega, Madison, WI, USA).
2. Genomic DNA that will be interrogated, purified from tissue, plasma, FFPE, or other sources.

3. Standard PCR oligonucleotides (from vendors such as Integrated DNA Technologies, Inc., Coralville, IA, USA). For full- and ice-COLD-PCR formats, the T_m of these primers must be such that they do not generate amplicons at the temperature used for heteroduplex formation (usually 70.0 °C).
4. Polymerase system (such as GoTaq Flexi DNA Polymerase system, Promega, Inc.; HF-2 *Taq* polymerase, BD-Clontech, Inc.; Jumpstart *Taq* polymerase, Sigma-Aldrich; or Phusion high fidelity polymerase, Finnzymes Inc.).
5. Deoxyribonucleotide triphosphate (dNTP) mix.
6. Fluorescent dye (such as LCGreenPlus+, Idaho Technologies, Inc. or SYBR Green, Invitrogen, Inc.). PCR in real time is not required for COLD-PCR, but can be helpful to identifying the T_c.
7. A Smart Cycler II real-time thermocycler (Cepheid, Inc.) is used at our laboratory; however, other real-time or standard PCR thermocyclers with high temperature precision and reproducibility may be used.
8. Optionally, Light Scanner HR96 system (Idaho Technologies, Inc.) can be utilized for high resolution melt (HRM) analysis, to prescreen the samples before Sanger sequencing. Any real-time PCR thermocyclers that incorporates the HRM option can also be employed.
9. If ice-COLD-PCR is used: a 3′-phosphorylated oligonucleotide complementary to the wild type strand is required. Other 3′ end modifications that prevent polymerase extension can be used as well. Also, a maximum overlap of the reference sequence with the priming region should be no more than ~5 bp, so that the primers do not bind to it.

3 Methods

3.1 Procedure: Identification of the Critical Denaturation Temperature

The T_c should be determined experimentally for each COLD-PCR amplicon.

1. Perform real-time melting analysis in the same instrument and reagents that will be used for COLD-PCR. In our laboratory, this is done in the presence of LCGreenPlus+ dye on a Smart Cycler II real-time PCR thermocycler, to determine the T_m of a wild type amplicon first or the T_m of the hybridized duplexes in ice-COLD-PCR (*see* Subheading 3.6), *see* also **Note 1**. A good initial approximation to the T_c is then given by the empirical formula $T_c = T_m - 1.0$.
2. To fine-tune the choice of T_c, a series of denaturation temperatures at 0.5 °C decrements below the amplicon T_m should be evaluated. The appropriate T_c will be the lowest temperature

that reproducibly yields a high-quality PCR product and provides good enrichment. The optimal T_c value may be dependent on the amplicon size (typically, amplicons smaller than 200 bp are required), GC content, and reagents composition (*see* **Note 2**).

3. For the TT-COLD-PCR approach, the determination of the exact T_c is not necessary, but the T_m of the amplicon still has to be derived experimentally to establish the appropriate cycling protocol.

3.2 COLD-PCR Reagent Conditions and Concentrations

Use the same standard reagent conditions in COLD-PCR or TT-COLD-PCR reactions as used in conventional PCR. The use of a fluorescence dye (such as LCGreen+) allows the PCR to be monitored in real time. Although any polymerase system can be employed, the use of a high fidelity enzyme such as Phusion™ high fidelity DNA polymerase with reported error rates of 4.2×10^{-7} is recommended. Final reaction concentrations usually applied in our laboratory are as follows:

1. 1X Phusion™ Buffer that contains 1.5 mmol/L $MgCl_2$ (or other 1× PCR Buffer with the incorporation of 1.5–4 mmol/L $MgCl_2$).
2. dNTP mix: 0.2 mmol/L (each).
3. Primers (upstream and downstream): 0.1–0.5 μmol/L (each).
4. LCGreenPlus+dye: 0.1–1×.
5. Phusion™ DNA polymerase: 0.5 U (or any DNA polymerase system).
6. Template DNA: If the template is genomic DNA, 10–100 ng is used as input. If the template is a PCR product from a first PCR reaction; 1:1,000–1:10,000 dilution is usually used as input template for the COLD-PCR reaction.
7. Balance to 25.0 μl with DNAse, RNAse-free water.
8. If ice-COLD-PCR is performed: Oligonucleotide reference sequence: ~25 nmol/L.

3.3 PCR Thermocycling Conditions

COLD-PCR thermocycling conditions should be designed as either fast-, full-, or ice-COLD-PCR depending upon the type of mutation enrichment that is desired, as discussed in more detail below (*see* Subheading 3.5). If multiple amplicons in independent reactions need to be tested under the same cycling conditions, or when the exact T_c is unknown, the temperature-tolerant (TT)-COLD-PCR approach would be appropriate. Thermocycling protocols for all COLD-PCR formats are presented below.

1. *Fast-COLD-PCR thermocycling protocol*: Initial denaturation: 98.0 °C for 30 s, then ~5 cycles conventional PCR when using

a preamplified PCR product as a template (if genomic DNA template is used, perform conventional PCR until 3 cycles before the real-time PCR threshold, C_T-3 cycles) as follows: 98.0 °C for 10 s, annealing at 52.0–65.0 °C for 20 s and extension at 72.0 °C for 10 s. Then, automatically switch to COLD-PCR conditions, 25–35 cycles of denaturation at critical denaturation temperature (T_c) for 10 s, annealing at 52.0–65.0 °C for 20 s, and extension at 72.0 °C for 10 s (*see* **Notes 3** and **4**).

2. *Full-COLD-PCR thermocycling protocol*: Initial denaturation: 98.0 °C for 30 s, followed by ~5 cycles conventional when using a preamplified PCR template (if genomic DNA template is used, 25 cycles or $\approx C_T$) at 98.0 °C for 10 s, annealing at 52.0–65.0 °C for 20 s and extension at 72.0 °C for 10 s. Then, 25–35 cycles of denaturation at 98.0 °C for 10 s, heteroduplex formation (cross-hybridization) at 70.0–72.0 °C for 30 s, T_c for 10 s, annealing at 52.0–65.0 °C for 20 s, and extension at 72.0 °C for 10 s (*see* **Notes 4** and **5**).
3. *ice-COLD-PCR thermocycling protocol*: The protocol is similar to full-COLD-PCR (refer to above protocol). There is a cross-hybridization step at 70.0 °C where both the mutant and wild type alleles bind to the reference sequences, forming either a homoduplex or heteroduplex (*see* **Notes 4**, **6**, and **7**).
4. *Temperature-tolerant (TT)-COLD-PCR thermocycling protocol*: A single cycling protocol will amplify and simultaneously enrich DNA variants on various amplicons. Fast-, full-, and ice-COLD-PCR formats can be adapted to this protocol. The lowest T_m for all the amplicons that will be analyzed is the minimum T_m (T_{mmin}) (*see* **Note 8**).
   - *Fast-TT-COLD-PCR*: Initial denaturation: 98.0 °C for 30 s, followed by ~5 cycles when using a preamplified PCR template (if genomic DNA template is used, C_T-3 cycles) at 98.0 °C for 10 s, annealing at 52.0–65.0 °C for 20 s, and extension at 72.0 °C for 10 s. Then, 5–10 cycles of denaturation at the first critical denaturation temperature (T_{c1} normally, $T_{c1} = T_{mmin} - 2$ °C) for 20 s, annealing at 52.0–65.0 °C for 20 s, and extension at 72.0 °C for 10 s, followed by 5–10 cycles of denaturation at the second critical denaturation temperature (T_{c2}, $T_{c2} = T_{c1} + 0.3$ °C) for 20 s, annealing at 52.0–65.0 °C for 20 s, and extension at 72.0 °C for 10 s, repeated successively until a 1.5–2.0 °C temperature window is covered.
   - *Full-TT-COLD-PCR*: Initial denaturation: 98.0 °C for 30 s, followed by ~5 cycles when using a preamplified PCR template (if genomic DNA template is used, 25 cycles or $\approx C_T$) at 98.0 °C for 10 s, annealing at 52.0–65.0 °C for

20 s, and extension at 72.0 °C for 10 s. Then, 5–10 cycles of denaturation at 98.0 °C for 10 s, heteroduplex formation (cross-hybridization) at 70.0–72.0 °C for 30 s, first critical denaturation temperature (T_{c1} normally, $T_{c1} = T_{mmin} - 2.0$ °C) for 10 s, annealing at 52.0–65.0 °C for 20 s, and extension at 72.0 °C for 10 s. This is followed by consecutive 5–10 cycles for the next critical denaturation temperature (T_{c2}, $T_{c2} = T_{c1} + 0.3$ °C), repeated in consecutively until a 1.5–2.0 °C temperature window is spanned.

- *ice-TT-COLD-PCR*: Follow the same protocol as for TT-full-COLD-PCR.

3.4 Guidelines for the Design of COLD-PCR Amplicons and Primers

1. Choose a target PCR amplicon that possesses preferentially a single melting domain. The amplicon melting profile can be predicted by using DNA melting prediction software tools such as uMELT developed by the Wittwer lab (http://www.dna.utah.edu/umelt/um.php). If the target amplicons have multiple melting domains, the amplicon should be split and primers should be designed to amplify single melting domains. The presence of a single melting domain can also be tested experimentally by doing a real-time PCR in the presence of an intercalating dye, followed by melting analysis.
2. Enrichment of mutations via COLD-PCR is more efficient for amplicons smaller than 200 bp, as single base variations will have a larger effect on the T_m [11, 12].
3. The degree of enrichment obtained with COLD-PCR will be variable depending on amplicon size, DNA sequence, mutation location, type of DNA variation, and experimental conditions; however, in all cases the use of COLD-PCR followed by downstream detection methods is expected to improve the current limit of detection for minority alleles. In difficult cases such as A>T mutations in the vicinity of GC-rich sequences, the use of LNA-assisted ice-COLD-PCR [13] can provide satisfactory enrichment.
4. We have demonstrated mutation enrichment by ~1 order of magnitude (range, 6–22-fold) for mutant alleles in amplicons ~100–200 bp in length, and enrichment of up to ~2 orders of magnitude for shorter amplicons <100 bp [6, 15–17]. For deletions and/or insertions of 3 bp or more, the enrichment can be greater than 2 orders of magnitude [6].
5. Primer design guidelines for COLD-PCR are the same as for conventional PCR; however, the T_m for each primer should not be higher than 65.0 °C for full-COLD-PCR or ice-COLD-PCR because it may affect the formation of heteroduplex molecules during the hybridization step (if primers anneal at

70.0 °C, polymerase may extend and cause problems). In the event the target region has a high melting temperature, the heteroduplex formation step can be performed at temperatures slightly higher than 70.0 °C (for instance, at 72.0 °C).

3.5 Guidelines for the Selection of Full-, Fast-, Ice-, or Temperature-Tolerant-COLD- PCR

1. Whether to perform full-, fast-, ice-, or temperature-tolerant (TT)-COLD-PCR depends on the DNA variant likely to be present, the number of targets of interest and the desired mutation enrichment. For enriching insertions, deletions, and single base substitutions that result in either a comparable T_m or increase the T_m, full-COLD-PCR or ice-COLD-PCR [13] should be applied. For example, T:A→A:T, C:G→G:C will result in a comparable T_m, while T:A→G:C and T:A→C:G will increase the T_m. Conversely, for insertions, deletions, and single base substitutions (such as G:C→A:T or G:C→T:A) that lower the amplicon melting temperature, fast-COLD-PCR will be suitable to achieve better enrichment. Getting experience with fast-COLD-PCR is highly recommended for first-time users of COLD-PCR, as it is simple and results in substantial mutation enrichment with minimal effort involved.
2. If multiple targets need to be screened or if the exact T_c has not yet been determined, a temperature-tolerant approach in a full-, fast-, or ice-COLD-PCR format is advisable. TT-COLD-PCR allows the interrogation of multiple genomic regions by using a single cycling protocol.

3.6 Guidelines for the Determination of T_m and T_c of the Amplicon

1. The establishment of the amplicon T_m can be done by a melt curve analysis after real-time PCR amplification performed on wild type DNA under conventional conditions in the presence of an intercalating dye. Differences in the T_m values may be observed between instruments, buffer compositions, or reagent concentrations. Every time there is a change, T_m values should be redetermined (Fig. 2).
2. Ice-COLD-PCR: In this case, the T_m of the reference sequence will differ from that of the amplicon as it is shorter in length. The experimental determination of the T_m is done by using primers that generate an amplicon (wild type) of the same length and sequence as the reference sequence. Then, the T_m determination for the hybridized duplexes is by conventional PCR followed by a melt curve analysis of the RS amplicon duplex.
3. To fine-tune and define the optimal T_c of a target amplicon, test samples containing 10 % dilutions of DNA with known mutations (typically from commercial cell lines or clinical samples) in wild type DNA background can be used. This enables the assessment of the mutation abundance before and after COLD-PCR, in addition to the mutation enrichment (*see* **Note 9**).

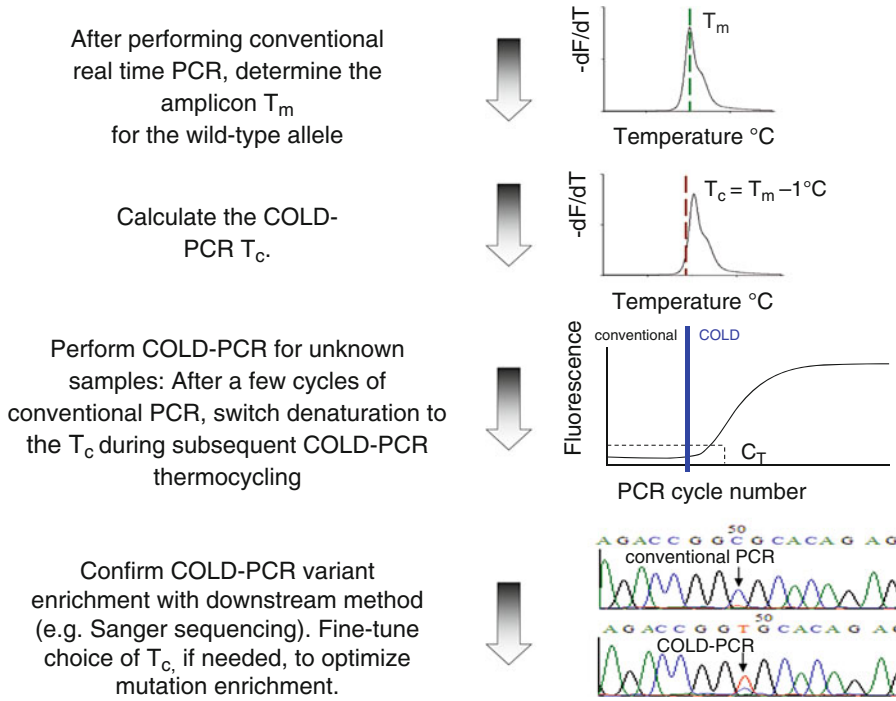

Fig. 2 A general overview of the optimization process for COLD-PCR is presented. The T_m is the amplicon melting temperature, as determined by –dF/dT plots (the negative derivative of fluorescence over temperature) and it is obtained by a post-real-time PCR melt curve analysis of the wild type amplicon; the T_c is the critical denaturation temperature employed in COLD-PCR. The C_T is the threshold cycle of the PCR amplification curve, for real-time PCR-based embodiments

4. As described in Subheading 3.3, fine-tuning of the T_c for fast-, full-, or ice-COLD-PCR should be performed as follows: (1) amplification of 10 % mutant dilution under conventional denaturation conditions (~95.0–98.0 °C), (2) change the denaturation temperature to a T_c of 0.5 °C below the amplicon T_m, (3) T_c = amplicon T_m – 1.0 °C, and (4) T_c = amplicon T_m – 1.5 °C. Next, evaluation of mutant enrichment according to the temperature gradient above can be predetermined by post-PCR high resolution melt (HRM) curve analysis and further confirmed by Sanger sequencing (*see* **Note 10**).
5. Alternatively the enriched mutant fraction can be determined using a restriction fragment length polymorphism (RFLP) assay that differentiates between mutant and wild type alleles, followed by gel, dHPLC, or capillary electrophoresis, if an appropriate restriction endonuclease is available [18].
6. The optimal T_c should be selected on the basis of the lowest denaturation temperature capable of still generating a reproducible PCR amplicon. To date, we have observed that for the majority of amplicons tested, $T_c = T_m - 1.0$ °C. The Ferrari/

Cremonesi group has reported a PCR-gradient-based approach to determine the optimal T_c (*see* **Note 11**), which is not dependent on real-time PCR [19, 20]. This can be used for non-real-time PCR thermocyclers.

3.7 Guidelines for the Determination of the Temperature Window for Temperature Tolerant COLD-PCR

1. The first step is to determine the melting temperatures (T_m) of the amplicons that will be screened, as previously described (Subheading 3.6).
2. TT-fast, TT-full-, or TT-ice-COLD-PCR protocols can be applied depending on the type of variant that is to be detected (*see* Subheading 3.5).
3. Typically, temperature windows spanning 1.5–2 °C can be applied, although larger temperature windows in bigger temperature increments during cycling are also possible [14]. This temperature range can be adapted in a step-wise manner, with consecutive steps of 5–10 cycles at each step, incrementing 0.3 °C in each step. The first T_c of the cycling protocol should be ≥1.5–2 °C below the lowest melting temperature of the amplicons tested [minimum T_m, $T_c = T_{mmin} - (1.0–2.0\ °C)$] (Fig. 3).
4. Additionally, if amplicons with higher T_ms have to be evaluated by using the same cycling conditions, but in different wells of a single thermocycler, variable DMSO/formamide/other organic solvent concentrations can be added within each reaction (1–5 % of the total reaction volume in order to lower the amplicon T_m) so that their final T_m is within the temperature range selected.

3.8 Selecting the Proper Thermocycler

1. When applying fast-, full-, or ice-COLD-PCR formats, the degree of mutant enrichment is highly dependent upon a precise T_c. A variance of ~0.5 °C in T_c may result in either failed amplification or poor enrichment (*see* **Note 12**). It is important that the selected thermocycler is highly precise in temperature such that there is little well-to-well variation. The SmartCycler II (Cepheid, Inc.) is excellent for optimization of thermocycling conditions because each well is individually controlled and calibrated.
2. Air-cooled thermocyclers, such as the centrifugal Rotor-Gene Q (Qiagen, Inc.) and the rotary glass capillary systems such as the LightCycler 480 (Roche, Inc.) and the LightScanner 32 (Idaho Technologies, Inc.) can also be used. In some of them, the anticipated well-to-well variation is as little as 0.01–0.05 °C. Some of these systems provide high temperature precision, real-time monitoring, high-throughput sample processing, as well as post-PCR high resolution melting (HRM). The HRM platform is valuable in its ability to detect quickly and effortlessly the presence of a low-level mutant, as well as offer a potential indication of mutation enrichment based upon the

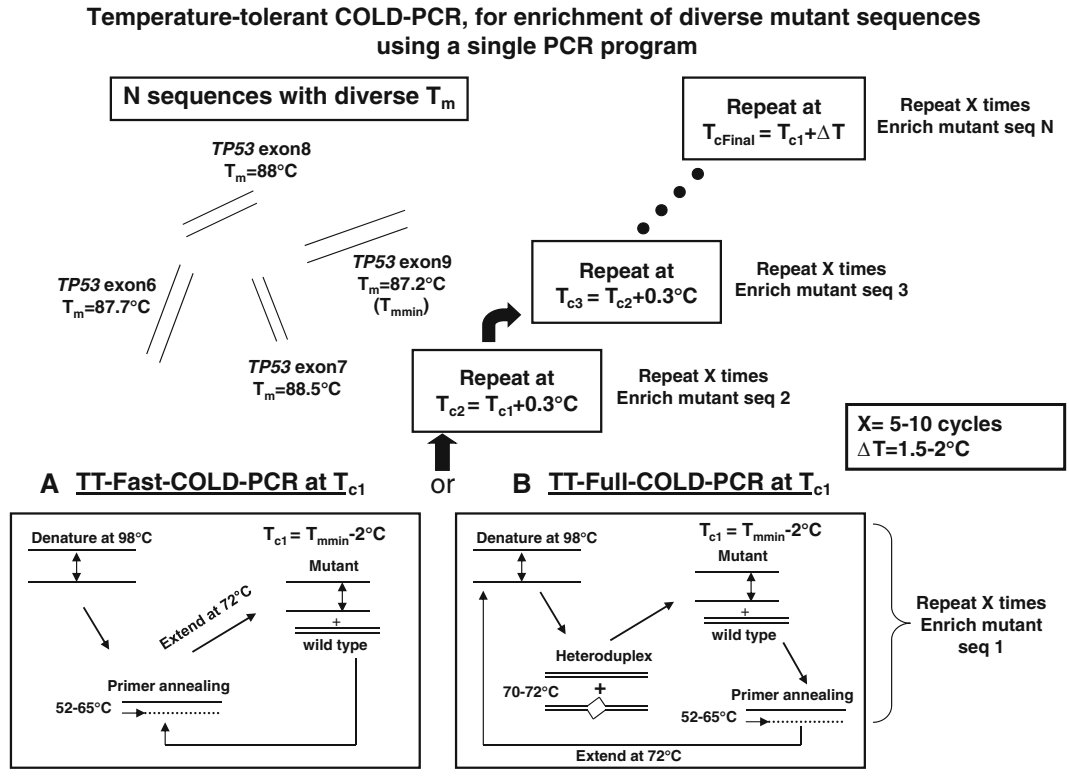

Fig. 3 *Temperature tolerant COLD-PCR*. Temperature tolerant (TT)-COLD-PCR protocol adapted to the two main forms of COLD-PCR, (**a**) fast- and (**b**) full-COLD-PCR for enrichment and amplification of multiple sequences under the same cycling protocol. TT-COLD-PCR (fast- or full-) is a step-up protocol where the different temperature steps are repeated 5–10 cycles each and the critical denaturation temperature is incremented by 0.3 °C at each step. The final temperature window that encompasses the different T_c of the targets of interest range from 1.5 to 3.0 °C, depending upon instrument limitations, polymerase system, or the formation of primer dimers. Adapted from Castellanos-Rizaldos et al. [14]

differential melt curve profiles relative to wild type control samples.

3. When the temperature tolerant approach is the protocol of choice, the temperature fluctuations observed between wells or different platforms do not severely affect the overall enrichment, so this relaxes somewhat the requirement for temperature stringency.

3.9 Assays Downstream of COLD-PCR

After COLD-PCR enrichment, several methods can be used to detect enriched minority alleles. Some of these downstream applications include:

1. *Sanger sequencing*: use this approach for both known and unknown mutation scanning and to determine the approximate degree of enrichment. It is broadly available; however, the

sensitivity is limited to detecting ~20 % mutant in a wild type background [21]. This mutation sensitivity limit can increase by 5–100-fold if one or two successive rounds of COLD-PCR are applied instead of conventional PCR [15, 18, 22].

2. *Pyrosequencing*: use this approach for scanning both known and unknown mutations. The detection sensitivity is around 5–10 % mutant alleles into wild type background [23]. COLD-PCR followed by pyrosequencing can identify mutations down to 0.5–1 % mutant in wild type DNA dilution, with mutation enrichments of 5–35-fold [6].
3. *MALDI-TOF genotyping*: use for high-throughput detection of known mutation; the detection sensitivity is around ~5–10 % mutant into wild type DNA [24]. The combination of fast-COLD-PCR with MALDI-TOF can increase this sensitivity by reliably detecting somatic mutations down to 0.1–0.5 % in wild type DNA background and provides mutation enrichments by 10–100-fold [7].
4. *TaqMan genotyping*: use for genotyping and quantifying known mutations; the sensitivity limit for mutation detection under conventional Taqman-PCR conditions is around 10 % mutant into wild type DNA [25–27]. The combination of this method with COLD-PCR improves the detection limit to 0.8 % of mutant DNA into wild type DNA under fast-COLD-PCR conditions in TaqMan format; and 0.1 % after two rounds of COLD-PCR-TaqMan [7]. The use of locked-nucleic acids/PNA modifications in the Taqman probe improves the mutation enrichment further (unpublished data).
5. *High Resolution Melting*: use for high-throughput and fast mutation scanning showing altered melting profiles compared to a wild type sequence [28]. Mutation detection limit is highly amplicon-dependent; however, COLD-PCR improves readily the detection limit, and most importantly, enables sequencing of the identified DNA variant. The application of COLD-PCR prior to HRM analysis instead of PCR under standard cycling conditions can accurately identify mutant mixtures as little as 0.1 % mutant DNA into wild type DNA [29].
6. *Next-generation sequencing* (*NGS*): high performance technology use for mutation detection; the reported sensitivity in this case is about ~2 % [15]. The enrichment of low abundance mutations by COLD-PCR before NGS, improves the sensitivity by allowing the detection of 0.02 % mutant alleles into wild type DNA background [15].

4 Notes

1. The amplicon T_m may differ when different PCR reagents are used (buffer composition and buffer batch, polymerase system, etc.), fluorescent dye type and concentration. Therefore, T_m values have to be derived experimentally in each case. It is important to keep track of the buffer lot number, as manufacturers occasionally change PCR buffer composition without warning. If the buffer changes, the optimal T_c has to be redetermined. It is important to consider that the efficiency of enrichment may be affected if an amplicon contains multiple melting domains and some mutant variants may not even enrich if they are located in the sequence between two melting regions.
2. We have observed that $T_c = T_m - 1.0$ °C is typically optimal for fast-COLD-PCR. However, this may vary depending on the amplicon and those that contain a high GC content normally present a T_c equivalent to the T_m of the amplicon—(0.5–0.8)°C.
3. Fast-COLD-PCR can be applied in two formats, either in a nested format from a larger PCR amplicon or directly from genomic DNA. In a nested fast-COLD-PCR reaction, a preliminary conventional PCR is performed. The PCR product generated is diluted (1:1,000–1:10,000) and subsequently used as a template for the fast-COLD-PCR reaction. Amplifying directly from genomic DNA is advantageous due to its simplicity; however, the reaction often reaches saturation before enough COLD-PCR cycles are applied. Thus the mutation enrichment can be smaller when amplifying directly from genomic DNA. One important consideration during the thermocycling protocol is when to begin the COLD-PCR cycling as the timing may determine the degree of enrichment achieved. In the nested format, ~5–10 cycles of amplification at a standard denaturation temperature are appropriate before beginning the COLD-PCR cycles. When amplifying directly from genomic DNA, amplification using the T_c should start ~3 cycles before the threshold cycle number (C_T) to accumulate a sufficient amount of DNA template. The C_T can be determined from the amplification profiles via real-time thermocycling or the PCR thermocycling program can be manually set to start the COLD-PCR cycles at a set C_T. Some real-time thermocyclers can be programmed such that specific cycling conditions automatically begin or end according to the C_T of the reaction, thus accommodating for sample-to-sample variation.
4. The number of initial cycles of conventional PCR is important. COLD-PCR cycling, using a certain T_c, must be initiated before the amplification growth curve reaches the reaction C_T.

5. Full-COLD-PCR can be applied similar to fast-COLD-PCR in a nested approach or, alternatively, directly from genomic DNA. Following conventional cycles, the PCR product is denatured at 98.0 °C then cross-hybridized at 70.0 °C for 30 s. We have found 70.0 °C is the ideal temperature for cross-hybridization; however, this hybridization temperature may be increased to 72.0 °C in case that the primers anneal at 70.0 °C (especially in those genomic regions where GC content is high). If this is the case, primer annealing and extension can occur during this step.
6. It is imperative to confirm the quality and purity of the 3′-modification on the oligonucleotide reference sequence for ice-COLD-PCR. If the reference sequence is not properly purified, it will be extended by the polymerase, resulting in wild type template amplification and therefore jeopardizing the mutation enrichment.
7. ice-COLD-PCR can be applied in a nested format as fast- or full-COLD-PCR. If this is the case, enough regular PCR cycles should precede ice-COLD-PCR conditions in order to generate heteroduplexed structures.
8. Temperature tolerant (TT)-COLD-PCR: In this case, the temperature window used may be as broad as 1.5–3.0 °C using finer T_c gradient, however, the number of cycles that can be performed is limited to the instrument, primer dimmer formation, polymerase inactivation, or reaction saturation. During TT-COLD-PCR, mutant enrichment will occur simultaneously for all amplicons, as long as their T_c falls within the T_c range examined, in individual tubes under the same cycling conditions.
9. Evaluation of mutant (or variant) DNA serial dilutions is important to allow the user to confirm both the successful amplification of the mutant fraction and enrichment of mid-, low-, and extremely low abundances of the mutant fraction.
10. If enrichment is not detected, confirm the empirical amplicon T_m and ensure that reagents and concentrations have not been altered.
11. Lower T_c can be evaluated in an attempt to increase mutation enrichment; however, if the T_c is too low, PCR will likely fail to amplify or will not be reproducible. The gradient approach described by Ferrari/Cremonesi [19, 20] is another alternative.
12. Well-to-well variation in thermocycling equipment can result in enrichment variability. Always ensure that the thermocycler is properly calibrated and shows consistent results.

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