COLD-PCR: a new platform for highly improved mutation detection in cancer and genetic testing

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Abstract
PCR is widely employed as the initial DNA amplification step for genetic testing and cancer biomarker detection. However, a key limitation of PCR-based methods, including real-time PCR, is the inability to selectively amplify low levels of variant alleles in a wild-type allele background. As a result, downstream assays are limited in their ability to identify subtle genetic changes that can have a profound impact on clinical decision-making and outcome or that can serve as cancer biomarkers. We developed COLD-PCR (co-amplification at lower denaturation temperature-PCR) [Li, Wang, Mamon, Kulke, Berbeco and Makrigiorgos (2008) Nat. Med. 14, 579–584], a novel form of PCR that amplifies minority alleles selectively from mixtures of wild-type and mutation-containing sequences irrespective of the mutation type or position on the sequence. Consequently, COLD-PCR amplification from genomic DNA yields PCR products containing high-prevalence variant alleles that can be detected. Since PCR constitutes a ubiquitous initial step for almost all genetic analysis, COLD-PCR provides a general platform to improve the sensitivity of essentially all DNA-variation detection technologies including Sanger sequencing, pyrosequencing, single molecule sequencing, mutation scanning, mutation genotyping or methylation assays. COLD-PCR combined with real-time PCR provides a new approach to boost the capabilities of existing real-time mutation detection methods. We replaced regular PCR with COLD-PCR before sequencing or real-time mutation detection assays to improve mutation detection-sensitivity by up to 100-fold and identified novel p53/Kras/EGFR (epidermal growth factor receptor) mutations in heterogeneous cancer samples that were missed by all existing methods. For clinically relevant micro-deletions, COLD-PCR enabled exclusive amplification and isolation of the mutants. COLD-PCR is expected to have diverse applications in the fields of biomarker identification and tracing, genomic instability, infectious diseases, DNA methylation testing and prenatal identification of fetal alleles in maternal blood.

Introduction
PCR plays a key role in molecular diagnosis and in the detection of mutations. A commonly encountered situation is when variant DNA sequences exist in the presence of a large majority of wild-type alleles, such as when DNA is obtained from heterogeneous cancer biopsies. As commonly applied, PCR does not contain an inherent selectivity towards variant (mutant) alleles; thus both variant and non-variant alleles are amplified with approximately equal efficiency. The burden of identifying and sequencing the mutation in a PCR product falls on downstream assays. Despite being reliable for screening germline or prevalent somatic mutations, sequencing of unknown low-prevalence mutations using these otherwise powerful technologies is still problematic. The significance of identifying these mutations, however, is critical in several fields of medicine, including cancer, prenatal diagnosis and infectious diseases [1–3]. We recently developed COLD-PCR (co-amplification at lower denaturation temperature-PCR), a new form of PCR that preferentially enriches ‘minority alleles’ from mixtures of wild-type and mutation-containing sequences, irrespective of where an unknown mutation lies. Consequently, COLD-PCR amplification from genomic DNA yields PCR products containing high percentages of variant alleles, thus permitting their detection. Since PCR constitutes a common initial step in almost all genetic analysis, COLD-PCR provides a general platform to improve the sensitivity of essentially all DNA-variation detection technologies, including Sanger sequencing, pyrosequencing, real-time PCR, mutation scanning, mutation genotyping and methylation assays (Figure 1).

Principle of COLD-PCR
The principle of COLD-PCR is described in Figure 2. A single nucleotide mismatch anywhere along a double-stranded DNA sequence generates a small but predictable change to the ‘melting’ temperature of DNA ($T_m$) for that sequence [4,5]. Depending on the sequence context and
position of the mismatch, $T_m$ changes of $0.2–1.5\,^{\circ}\mathrm{C}$ are common for sequences up to 200 bp or higher [4,5]. We observed the following: (i) for each DNA sequence, there is a critical denaturation temperature ($T_c$) that is lower than $T_m$ and below which PCR efficiency decreases abruptly. For example, a 167 bp p53 sequence used in the present study amplifies well when PCR denaturation temperature is set to 87 $\,^{\circ}\mathrm{C}$, amplifies modestly at 86.5 $\,^{\circ}\mathrm{C}$ and yields no detectable product when PCR denaturation is set to 86 $\,^{\circ}\mathrm{C}$ or below. Therefore, in this example, $T_c \approx 86.5\,^{\circ}\mathrm{C}$. (ii) The critical denaturation temperature ($T_c$) is strongly dependent on DNA sequence. DNA amplicons differing by a single nucleotide result reproducibly in different amplification efficiencies when PCR denaturation temperature is set to $T_c$. This new observation can be exploited during PCR amplification for selective enrichment of 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; hetero-duplexes, 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. 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 operation of full and fast COLD-PCR, for example a 167 bp p53 sequence, is described in Figures 2(A) and 2(B) respectively.

Applications of COLD-PCR in conjunction with specific downstream assays

Improvement of enzymatic mutation detection via COLD-PCR

To validate COLD-PCR, we used serial dilutions of DNA from tumour-derived cell lines containing mutations or micro-deletions at different positions along p53 exon 8 and Kras exon 2 (codons 12–13), or samples with micro-deletions in EGFR (epidermal growth factor receptor) exon 19. In addition, genomic DNA from a series of colon and lung cancer surgical specimens and plasma-circulating DNA collected under IRB (institutional review board) approval were utilized for validation on clinical specimens. For each amplicon, a single $T_c$ was used to enrich mutations at all positions simultaneously. For an initial evaluation of the mutation enrichment obtained via COLD-PCR, mutation-containing DNA was mixed with wild-type DNA, amplified using the COLD-PCR programme described in Figure 2(A) or 2(B), and the PCR products were digested with restriction enzymes that recognize selectively either only the mutation-containing DNA or only the wild-type DNA. When no restriction enzyme that distinguishes between mutant and wild-type was available, the Surveyor™ nuclease that digests mismatch-containing DNA was used instead [6,7]. The digested products were then examined via dHPLC (denaturing HPLC). For comparison with COLD-PCR, identical experiments were conducted after regular PCR. Overall, as shown in the original publication, our results indicated a major (~10–20-fold) improvement in the sensitivity of RFLP (restriction-fragment-length polymorphism; restriction enzymes for specific mutations) and of unknown mutation scanning (Surveyor™ nuclease) by replacing regular PCR with full or fast COLD-PCR.

Improvement of sequencing technologies via COLD-PCR

To examine the impact of mutation enrichment on the sensitivity of sequencing technologies, PCR products obtained after COLD-PCR or regular PCR were processed for Sanger dideoxy-terminator sequencing. When COLD-PCR at a $T_c$ of 86.5 $\,^{\circ}\mathrm{C}$ was applied to the 167 bp exon 8 fragment, the enrichment was evident for all p53 mutations tested. For example, DNA from HCC2218 cells (C→T mutation), initially diluted in wild-type cells down to 5% mutant/wild-type ratio, becomes ~65% mutant/wild-type ratio after COLD-PCR, as estimated by observing the sequencing chromatograms (i.e. an enrichment of the mutation by ~13-fold). Figure 3(A) depicts a representative Sanger sequencing result. All mutations detected via COLD-PCR, which were undetectable via conventional sequencing, were also independently cross-verified using RFLP methods [8]. Wild-type p53 samples, amplified multiple times via COLD-PCR, indicated no mutation [8], thus excluding the possibility of artefacts generated via polymerase-introduced errors. Overall, for all of the p53 mutations studied for this 167 bp fragment, the mutation enrichment varied by ~5–13-fold depending on the mutation position.

Pyrosequencing is another sequencing technology that could benefit by incorporating COLD-PCR. The current detection limit for pyrosequencing is ~10% mutant/wild-type ratio [9]. We repeated the comparison of regular PCR with COLD-PCR for several of the p53 exon 8 and Kras codon 12/13 mutations also examined via Sanger sequencing.
Figure 2 | Description of COLD-PCR, for example a 167 bp p53 sequence

(A) After a number of regular PCR cycles that enable initial build-up of target amplicon(s), the PCR programme is switched to full COLD-PCR. After denaturation at 94°C, the PCR amplicon(s) are allowed to cross-hybridize at an intermediate temperature (e.g. 70°C for 2–8 min). Because mutant alleles are in a minority, most mutant alleles end up in a mismatch-containing structure (‘heteroduplex’) that has a lower $T_m$ than the fully matched structure (‘homoduplex’). Next, the PCR temperature is raised to the $T_c$ to denature the mismatch-containing sequences preferentially over the fully matched sequences. Finally, the temperature is reduced to 55°C to allow primers to bind and replicate the preferentially denatured sequences. Because critical denaturation is performed at every PCR cycle, the differential enrichment of mutation-containing alleles is compounded exponentially, and results in a large difference in overall amplification efficiency between mutant and wild-type (w. type) alleles, at the end of the cycling. (B) Fast COLD-PCR: preferential amplification of mutations via COLD-PCR is so pronounced that, for most of the point mutations, mutant enrichment occurs even without performing the intermediate cross-hybridization step at 70°C. Thus rapid PCR amplification performed at the $T_c$ instead of 94°C discriminates strongly towards the lower-$T_m$ allele. For example, if two alleles differing by a single base-pair, A:T instead of G:C, are present, the A-allele is enriched preferentially during cycling, presumably because the A:T amplicon has a slightly lower $T_m$ than the G allele. For mutation enrichment to occur, the full COLD-PCR protocol requires the build-up 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 build-up, 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 programme described in (A) is necessary. By applying the full COLD-PCR programme, 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 $T_m$.

### A. FULL COLD-PCR (FOR ENRICHMENT OF ALL MUTATIONS)

- **Denature DNA**
  - 94°C
- **Reduce temperature**
  - Cross-hybridize mutant with wild type sequences
- 70°C
- **Selectively denature the mismatched sequences at critical temperature ($T_c$=86.5°C)**
  - Mutant **$T_c$=86.5°C**
  - W. type
- Reduce temperature for primer annealing
- Extend at 72°C
- Return to 94°C

**Preferentially copy the mutated sequences**

### B. FAST COLD-PCR (FOR ENRICHMENT OF $T_m$-REDUCING MUTATIONS)

- **Denature DNA**
  - 94°C
- **Reduce temperature**
  - FOR PRIMER ANNEALING
  - 55°C
- Extend at 72°C

**Preferentially copy the mutated sequences**

Serial dilutions of DNA from mutant cell lines indicated that COLD-PCR pyrosequencing could identify mutations down to a prevalence of 0.5–1% for the mutations examined [8], and mutation enrichments of 5–35-fold were obtained, compared with regular PCR pyrosequencing. Screening of DNA from three of the clinical samples demonstrated that COLD-PCR pyrosequencing revealed low-level p53 codon 273 and the Kras mutations that were missed when using regular PCR.
Figure 3 | COLD-PCR improves mutation detection by downstream assays

(A) Improvement of Sanger sequencing via COLD-PCR. DNA containing mutations in p53 exon 8 (HCC2218 cell line; 14516 C > T) was diluted in wild-type DNA and amplified using COLD-PCR at a single $T_c$ ($T_c = 86.5^\circ C$) to obtain a 167 bp PCR product (lower panel). Sanger dideoxy-terminator sequencing using the forward primer was applied to the PCR products. Regular PCR sequencing was also performed on the same samples (upper panel), for comparison. Arrows refer to the mutated nucleotide. 

(B) Improvement of pyrosequencing via COLD-PCR. DNA from cell line A549 was diluted 33-fold into wild-type DNA, and a 98 bp Kras exon2 segment was amplified via COLD-PCR (lower panel) ($T_c = 80^\circ C$) or via regular PCR (upper panel), followed by pyrosequencing. The G>A mutation of the A549 cell line was only visible when COLD-PCR was applied. Arrows refer to the mutated nucleotide.

(C) COLD PCR improves the sensitivity of genotyping technologies. Mass spectra obtained after MALDI–TOF are depicted: 87 bp p53 exon 8 fragments using plasma-circulating DNA were amplified using fast COLD-PCR ($T_c = 83.5^\circ C$) and genotyped using MALDI–TOF, to detect the ‘hotspot’ mutation at p53 codon 273. The G > A mutation was detectable using COLD-PCR–MALDI–TOF (lower panel). In contrast, regular PCR–MALDI–TOF was not able to detect the ‘hotspot’ p53 mutation (upper panel). 

(D) COLD-PCR improves the sensitivity of TaqMan genotyping technologies. Serial dilutions of H1975 cell line DNA containing T790M mutation of EGFR exon 20 in wild-type DNA were screened with regular or COLD-PCR Taqman genotyping for T790M mutation. Upper panel: regular PCR Taqman genotyping for T790M mutation (detection limit = 12% mutant/wild-type ratio). Lower panel: COLD-PCR Taqman genotyping for T790M mutation (detection limit = 0.8% mutant/wild-type ratio).

Pyrosequencing. A typical example demonstrating the improvement of pyrosequencing after COLD-PCR is depicted in Figure 3(B). These low-level p53 mutations can be clinically relevant; therefore their detection is of significance. For example, p53 exon 8 mutations at codon 273 have been associated with bad prognosis in lung cancer [10,11]. Overall, our results indicate that both Sanger sequencing and pyrosequencing can identify clinically relevant low-prevalence somatic
mutations in tissue samples by replacing regular PCR with COLD-PCR. These mutations would have been missed after regular PCR sequencing or pyrosequencing.

**Improvement of genotyping technologies via COLD-PCR**

High-throughput genotyping technologies such as MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) have been proposed for detection of somatic mutations in clinical samples [12]. The sensitivity limit of MALDI–TOF for low-level mutation detection is ∼5–10% [12]. However, the prevalence of mutations in heterogeneous tumour specimens or in DNA from plasma can be of the order of 5% or lower [7], which poses questions on the reliability of MALDI–TOF in these cases. We employed the same approach used for Sanger sequencing, to identify specific p53 exon 8 or Kras mutations using serial dilutions of mutation-containing cell lines into wild-type samples and by comparing COLD-PCR–MALDI–TOF with regular PCR–MALDI–TOF. Our results [8] indicated that mutation enrichments of 10–100-fold were obtained by replacing regular PCR with fast COLD-PCR before MALDI–TOF and that somatic mutations at a prevalence of 0.1–0.5% could be detected. The mutation enrichment caused by fast COLD-PCR was higher for samples containing the lowest mutant/wild-type ratios. When clinical samples containing low-prevalence somatic mutations were screened using COLD-PCR–MALDI–TOF, mutations in tumour samples and in plasma-circulating DNA were detectable (Figure 3C). Using regular PCR–MALDI–TOF, the mutations in these clinical samples were either barely detected or completely missed (Figure 3C).

Another technology employed for genotyping and somatic mutation detection is Taqman QRT-PCR (quantitative real-time PCR). We designed a 21 bp Taqman probe matching the mutant (T) allele in the EGFR exon 20 codon T790M drug-resistance mutation, while forming a G:T mismatch with the wild-type sequence. We then diluted genomic DNA from H1975 cells (heterozygous mutant) into wild-type DNA and performed QRT-PCR to test whether mutations can be identified at low mutation prevalence. COLD-QRT-PCR, or alternatively regular QRT-PCR, was performed directly from genomic DNA on a real-time PCR machine. The results in Figure 3(D) indicate that COLD-QRT-PCR magnifies the difference between mutant and wild-type samples and can detect much lower prevalence mutations than regular QRT-PCR.

**Selections of unknown deletions via COLD-PCR**

Micro-deletions (3–20 bp) of a known or variable sequence are an important group of mutations to identify. For example, 5–15 bp micro-deletions occurring at EGFR exon 19 confer a positive response on tyrosine kinase inhibitors in lung adenocarcinoma [13]. Application of full COLD-PCR to clinical samples and plasma-circulating DNA from lung adenocarcinoma patients identified traces of EGFR deletion of different sequences in exon 19 that were not possible to sequence using regular PCR [8]. The enhanced capability of identifying and sequencing traces of such deletions by replacing regular PCR with COLD-PCR should increase confidence in therapeutic decision-making based on molecular EGFR profiling of lung adenocarcinoma and allows improved detection of unknown deletions/insertions in other similar situations. Overall, deletion enrichments of at least 300-fold are obtained using full or fast COLD-PCR, and for Tm-reducing deletion mutants, deletion/wild-type ratios of 1:3000 can be directly sequenced.

**Conclusion**

Because in the forthcoming era of molecular medicine, clinical decisions will increasingly rely on molecular tumour profiling, the reliability of identifying somatic mutations in diverse clinical specimens, including heterogeneous tumours and bodily fluids, must be high. In agreement with earlier reports [7,14,15], our results indicate that Sanger sequencing, as well as pyrosequencing and MALDI–TOF, were deficient in detecting low-prevalence mutations in mixed tumour samples. Four of 43 surgical samples and 3 of 10 plasma samples tested contained clinically important p53, Kras or EGFR mutations that were not detected by any of these methods when preceded by regular PCR. Real-time (Taqman) PCR was also significantly improved by using COLD-PCR in real-time format. Replacement of regular PCR with COLD-PCR provides a ‘universal boost’ to these widely used technologies and enables them to be used with the required confidence in routine screening of cancer specimens for somatic mutations, including low-level mutation screening of surgical/FFPE (formalinfixed, paraffin-embedded) tumour samples or bodily fluids.

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**References**


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