

# COLD-PCR for early detection of hepatitis B virus antiviral drug resistance mutations

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## KEY MESSAGES

1. Co-amplification at lower denaturation temperature-PCR (COLD-PCR) was developed for early detection of hepatitis B virus (HBV) drug resistance mutations.
2. With a simple alteration of denaturation temperature in the thermal cycle, COLD-PCR could detect drug resistance mutations that existed at a level of 5-10% within a mixed pool, compared with a level of  $\geq 25\%$  for conventional PCR.
3. In patients prescribed lamivudine or telbivudine, COLD-PCR was more sensitive than conventional PCR, with a higher mutation detection rate.
4. COLD-PCR is useful for patient monitoring, as it is more sensitive than conventional PCR in early detection of drug resistance mutations.

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## Introduction

Nucleos(t)ide analogues (NAs) are effective therapeutic agents for the treatment of chronic hepatitis B virus (HBV) infection. However, long-term use of NAs is often hampered by the emergence of drug resistance mutations, causing potentially serious consequences such as liver decompensation and mortality. A more sensitive method for early detection of drug resistance mutations is needed.

Conventional PCR amplification of HBV DNA followed by direct sequencing of the purified amplicons for detection of drug resistance mutations has two advantages. First, it can detect any novel mutation within the amplicons. Second, it is relatively inexpensive. However, it cannot detect a low level of mutations that comprise  $< 20\%$  of the total viral population.

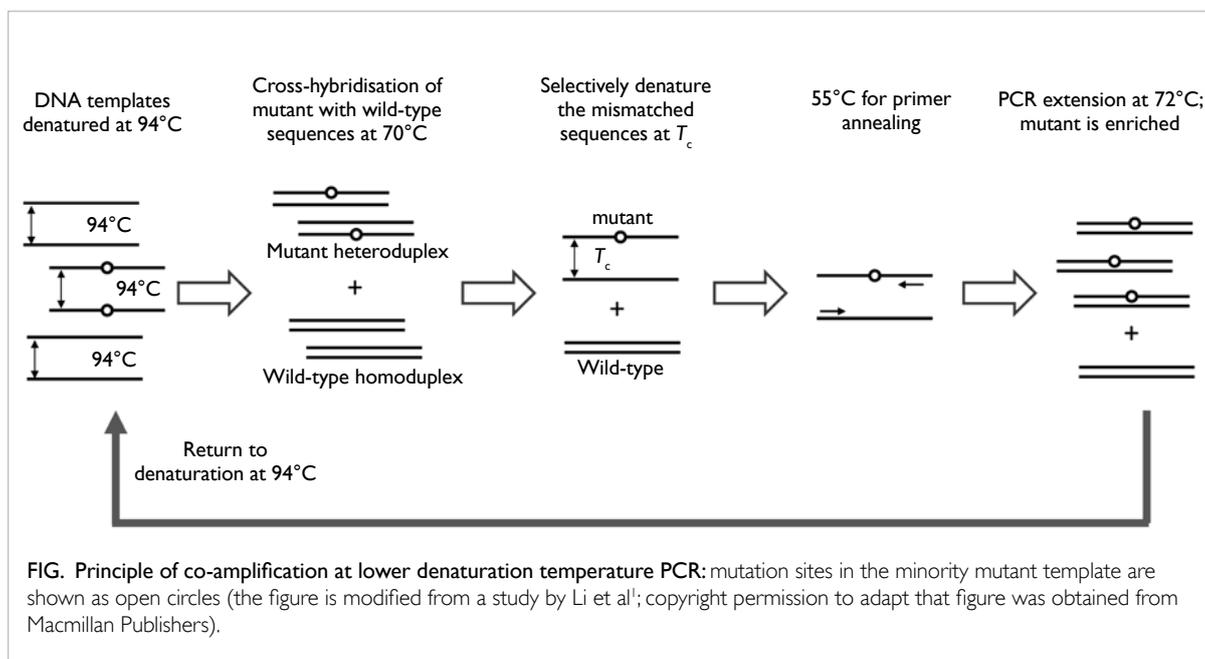
Co-amplification at lower denaturation temperature PCR (COLD-PCR) has been used for enrichment of a low level of variants within a mixed pool of sequences.<sup>1</sup> COLD-PCR relies on slight changes to the melting temperature ( $T_m$ ) in the DNA sequence caused by mutations within the sequence. For each DNA sequence, there is a critical denaturation temperature ( $T_c$ ) below which PCR efficiency decreases abruptly.<sup>1</sup>  $T_c$  is lower than  $T_m$  and is dependent on the DNA sequence itself. When the denaturation temperature of PCR is set to  $T_c$  (instead of the usual  $94^\circ\text{C}$ ), DNA amplicons with different mutations will have different amplification efficiencies. This property enables selectively enrich low-level mutations in a mixed pool.

The principle for COLD-PCR is shown in Fig 1. Like conventional PCR, COLD-PCR starts with a denaturation step at  $94^\circ\text{C}$ . Following denaturation, an intermediate hybridisation temperature of  $70^\circ\text{C}$  is used to promote cross-hybridisation of mutant and wild-type alleles, forming a heteroduplex. Then, with a denaturation temperature at  $T_c$ , the heteroduplex is selectively denatured and subsequently amplified, whereas the wild-type homoduplex does not amplify efficiently. As a result, mutations that exist in a minority are enriched by COLD-PCR and detected by subsequent sequencing.

This study aimed to (1) develop a modified COLD-PCR method to detect common HBV drug resistance mutations in patients undergoing lamivudine or telbivudine therapy (two of the licensed NAs) and (2) compare the performance of COLD-PCR with that of the LiPA HBV drug resistance assay (Fujirebio Europe, Belgium) and conventional PCR.

## Results

The execution of COLD-PCR depends on the experimental identification of  $T_c$ . Cloned wild-type HBV DNA and HBV DNA with representative drug resistance mutations were used as templates for PCR at different denaturation temperatures. Using a conventional denaturation temperature of  $94^\circ\text{C}$ , all wild-type and mutant sequences were amplified efficiently with Sybr-green-based real-time PCR, and experiments were performed with decreasing denaturation temperatures. PCR amplification was



not observed when the denaturation temperature  $<78^\circ\text{C}$ , which is defined as  $T_c$  when amplification efficiency decreases abruptly. Thus, the  $T_c$  for the PCR amplicon was determined to be  $78^\circ\text{C}$ .

Both COLD-PCR and conventional PCR were used to detect HBV mutations in mixtures of various proportions of cloned wild-type and mutant HBV DNA. Conventional PCR could detect drug resistance mutations only when the mutant plasmids existed at a level of  $\geq 25\%$  within the mixture, whereas COLD-PCR could detect drug resistance mutations at a level of 5-10% within the mixture.

The performance of the LiPA assay, COLD-PCR, and conventional PCR in detecting drug resistance mutations was compared in 106 patients treated with lamivudine and 30 patients treated with telbivudine. These patients have been followed up in our centre and previously reported to have virological breakthrough during follow-up.<sup>2,3</sup> Among these 136 patients, lamivudine/telbivudine-resistant mutations rtM204V/I were detected in 129 (95%), 108 (79%) and 84 (62%) patients by the LiPA assay, COLD-PCR, and conventional PCR, respectively. Drug resistance mutations were detectable by all three methods in 84 patients. To investigate whether these three methods can detect early drug resistance mutations, we attempted to detect rtM204V/I mutations from these 84 patients at 6-12 months before the previous mutation detection time points. In five samples taken from the earlier time point, HBV DNA was not amplifiable by PCR by any of the three methods. When analysing the samples taken

from both time points collectively, COLD-PCR was also more sensitive than conventional PCR: 35 (16%) samples had rtM204V/I detected by COLD-PCR but not by conventional PCR, while all samples with mutations detected by conventional PCR were detected by COLD-PCR.

## Discussion

Detection of drug resistance mutations is essential in the management of patients with antiviral therapy. Conventional PCR often cannot detect minority variants. In this study, COLD-PCR could detect HBV drug resistance mutations at a level of 5-10%, whereas conventional PCR could detect HBV mutations only at a level of  $\geq 25\%$ .

Among the three methods tested, the LiPA assay had the highest mutation detection rate in patients treated with lamivudine or telbivudine compared with COLD-PCR and conventional PCR. Among the samples with mutations detected by LiPA, COLD-PCR could detect mutations in more samples than conventional PCR. In particular, there were 35 samples with rtM204V/I mutations detected by COLD-PCR but not by conventional PCR, while all samples with mutations detected by conventional PCR were also detected by COLD-PCR. Thus, COLD-PCR was more sensitive than conventional PCR in detecting drug resistance mutations.

Although COLD-PCR may not be superior to the LiPA assay, it has several advantages. First, it is capable of enriching and detecting minority variants at all possible positions within the amplicon, whereas

LiPA is confined to a pre-defined set of variants. Second, compared with conventional PCR, it does not incur extra cost and is considerably cheaper than the LiPA assay. Third, compared with conventional PCR, COLD-PCR does not increase the run time of experiment.

Nevertheless, COLD-PCR has certain limitations. First, it may enrich minority species at other positions, thereby increasing the chance of obtaining high background sequencing noise. COLD-PCR may also induce a higher rate of PCR amplification error. This shortcoming can be overcome by using polymerases with proof-reading activities. In addition, as numerous possible quasispecies can be found in HBV clinical isolates, a single empirically determined  $T_c$  may not be applicable to all possible viral variations within the quasispecies population. Nevertheless, it is expected that this experimentally determined  $T_c$  would at least enhance the detection of minor mutations, and this was proven in the present study with both cloned plasmids and clinical specimen.

In a research setting, there are other more advanced methods to detect minor mutations, such as mass spectrometry, DNA microarray, next-generation sequencing, or ultradeep pyrosequencing. These methods are generally more expensive than COLD-PCR, especially when the number of samples tested is small. COLD-PCR is an affordable choice in the sensitive detection of minor mutations, especially in under-developed areas.

## Conclusion

This study demonstrated that COLD-PCR could sensitively detect HBV drug resistance mutations at a level of 5-10% of the total viral population. It is simple and inexpensive and has the advantage of detecting novel mutations along the HBV reverse transcriptase gene.

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