Chapter 7

Pyrosequencing®-Based Identification of Low-Frequency Mutations Enriched Through Enhanced-*ice*-COLD-PCR

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Abstract

A number of molecular diagnostic assays have been developed in the last years for mutation detection. Although these methods have become increasingly sensitive, most of them are incompatible with a sequencing-based readout and require prior knowledge of the mutation present in the sample. Consequently, coamplification at low denaturation (COLD)-PCR-based methods have been developed and combine a high analytical sensitivity due to mutation enrichment in the sample with the identification of known or unknown mutations by downstream sequencing experiments. Among these methods, the recently developed Enhanced-*ice*-COLD-PCR appeared as the most powerful method as it outperformed the other COLD-PCR-based methods in terms of the mutation enrichment and due to the simplicity of the experimental setup of the assay. Indeed, E-*ice*-COLD-PCR is very versatile as it can be used on all types of PCR platforms and is applicable to different types of samples including fresh frozen, FFPE, and plasma samples. The technique relies on the incorporation of an LNA containing blocker probe in the PCR reaction followed by selective heteroduplex denaturation enabling amplification of the mutant allele while amplification of the wild-type allele is prevented. Combined with Pyrosequencing®, which is a very quantitative high-resolution sequencing technology, E-*ice*-COLD-PCR can detect and identify mutations with a limit of detection down to 0.01 %.

Key words Pyrosequencing®, Genotyping, Mutation detection, E-*ice*-COLD-PCR, Real-time PCR, Mutation hotspots, *KRAS*

1 Introduction

Over the last 30 years, a number of molecular methods have been developed for the detection of mutations in different regions of interest such as mutation hotspots. It has become clear that one of the main clinical challenges is the capacity to detect mutations with very high sensitivity in all types of clinical samples for diagnosis, the choice of a personalized treatment regimen, and/or the monitoring of the treatment response. Among the available methods, some require a combination of PCR and sequencing technologies while others are generally used without any downstream sequencing experiments such as high-resolution melting (HRM)

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and allele-specific PCR (AS-PCR)-based methods [1, 2]. Assays using sequencing-based mutation detection have the advantage to allow the identification of the mutated base pair (in contrast to HRM) and they do not require prior knowledge of the mutation (in contrast to AS-PCR).

Therefore, Sanger sequencing has become the gold standard for mutation detection. However, Sanger sequencing has an average analytical sensitivity of only 20 % for the mutated alleles in a wild-type background [3]. Pyrosequencing® is a more recent quantitative real-time sequencing method that has also frequently been used for mutation detection [4, 5]. Pyrosequencing is based on the presence or absence of the incorporation of a nucleotide during primer extension [6, 7]. In contrast to Sanger sequencing, which relies on the random incorporation of fluorescent ddNTPs during primer extension steps, only one specific nucleotide is present at any time in the Pyrosequencing reaction. Following nucleotide incorporation, the released pyrophosphate (PPi) is used as substrate in combination with adenosine 5′ phosphosulfate (APS) by an ATP sulfurylase to produce ATP [4]. The latter is in turn used by luciferase to oxidize luciferin into oxyluciferin resulting in a light emission proportional to the amount of incorporated nucleotide [4]. The limit of detection of Pyrosequencing has been evaluated around 5 % for the mutant allele, which is thus far more sensitive than Sanger sequencing [3].

To further increase the limit of mutation detection, a large variety of PCR-based methods have been developed to enrich for unknown mutations in samples prior to sequencing [8]. For example, coamplification at lower denaturation temperature (COLD- or *full*-COLD) PCR has been developed to allow high enrichment of mutations located in the PCR amplification product (Fig. 1) [9].

The principle of this method is based on the formation of WT-WT homoduplexes and WT-mutant heteroduplexes at 70 °C after an initial denaturation step and followed by selective denaturation of the WT-mutant heteroduplexes at a critical temperature (Tc) determined as the melting temperature (Tm) of the amplicon minus one degree [9]. Then, primer annealing and elongation steps are performed where mutant and WT alleles undergo exponential or linear amplification, respectively, leading to enrichment of the mutated alleles. This protocol has been slightly modified in the *fast*-COLD-PCR assay where no initial denaturation step is performed and no mutant-WT heteroduplexes are formed (Fig. 1). *fast*-COLD-PCR enables a stronger enrichment of mutations compared to *full*-COLD-PCR for Tm-reducing mutations such as G:C>A:T or G:C>T:A, which are the most frequent occurring mutations [10, 11]. However, mutation enrichment is completely abolished for Tm-equivalent and Tm-increasing mutations in *fast*-COLD-PCR [10, 11]. Therefore a new COLD-PCR-based approach has been developed enabling a high enrichment of all

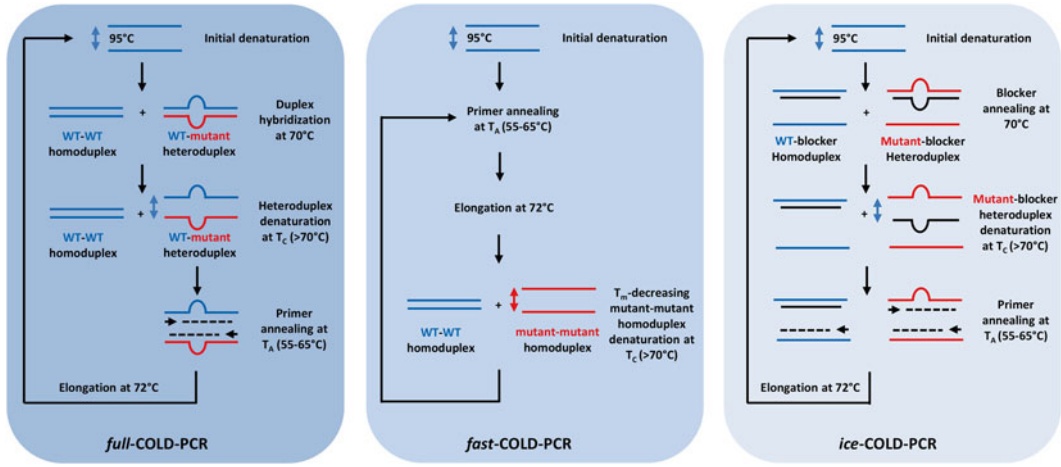

Fig. 1 Principle of COLD-PCR-based methods. In the *full*-COLD-PCR assay, mutation enrichment is performed after an initial denaturation step, followed by the formation of WT-WT homoduplexes and WT-mutant heteroduplexes at 70 °C, selective denaturation of WT-mutant heteroduplexes at the Tc and primer annealing and elongation. In a *fast*-COLD-PCR assay, WT-WT and mutant-mutant homoduplexes are formed after a first denaturation, annealing, and elongation cycle and enrichment of the mutation is then performed during PCR cycles including a selective denaturation of mutant-mutant homoduplexes at the Tc followed by primer annealing and elongation steps. In the *ice*-COLD-PCR assay, WT-blocker homoduplexes and mutant-blocker heteroduplexes are formed after an initial denaturation step followed by the selective denaturation of mutant-blocker heteroduplexes at the Tc and primer annealing and elongation resulting in mutation enrichment. Tc: critical temperature

types of mutations, called *i*mproved and *c*omplete *e*nrichment (*ice*)-COLD-PCR [11]. *ice*-COLD-PCR introduces a non-elongable blocker probe that is complementary to the WT sequence and that overlaps with five nucleotides at the 3′ end of each primer, which is used to block WT-homo- and mutant heteroduplex formation [11].

One of the drawbacks of COLD-PCR-based methods is the use of a critical temperature (Tc), which has to be very accurate as a slight variation of as little as 0.2 °C could completely abolish the mutation enrichment [11]. Moreover, *ice*-COLD-PCR requires two pairs of overlapping PCR primers to determine the correct Tc as well as a first PCR amplification [11]. Therefore, we developed Enhanced-*ice*-COLD-PCR (E-*ice*-COLD-PCR) as a modified version of *ice*-COLD-PCR which contains chemically modified nucleotides (locked nucleic acid (LNA) bases) in a primer-overlapping blocker probe (Fig. 2) [12].

The determination of the Tc requires only one primer pair and a strong mutation enrichment is achieved, which outperformed the originally reported approach. Efficient enrichment can be achieved over a large range of temperatures making the method easily usable on different thermocycler platforms [12]. Another advantage of E-*ice*-COLD-PCR is that it requires no pre-amplification PCR and

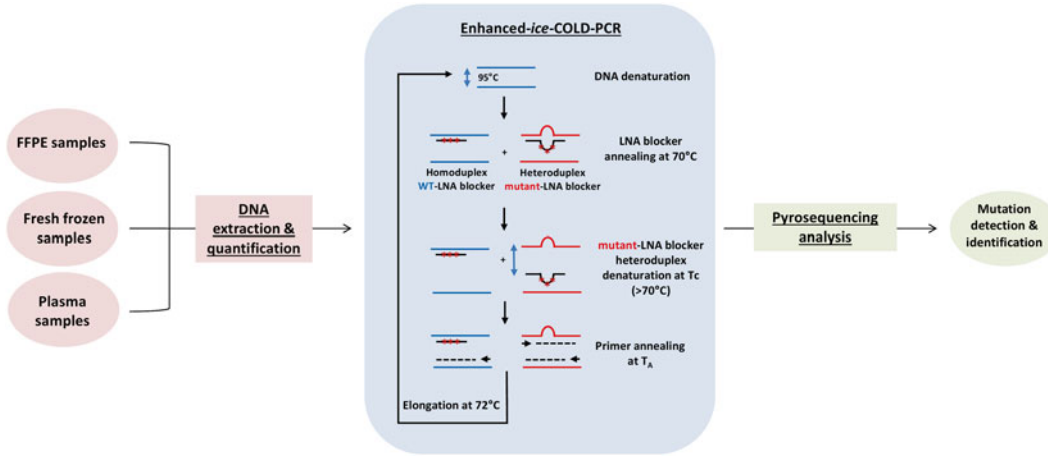

Fig. 2 General workflow for the enrichment, detection and identification of mutations by Enhanced-*ice*-COLD-PCR and Pyrosequencing. Different types of samples can be used for this method including DNA from FFPE, fresh-frozen, and plasma samples. After quality control, an E-*ice*-COLD-PCR reaction is performed using two PCR primers, one of them being biotinylated, flanking the region with the mutation (hotspot) of interest. The blocker probe complementary to the WT sequence allows the enrichment of the mutation during the PCR, performed with a cycling protocol in which only the mutant sequences undergo exponential amplification while the amplification of WT sequences remains linear. The resulting PCR product is purified prior to the Pyrosequencing analysis, which allows the accurate and ultra-sensitive detection and identification of mutations present in the analyzed region of interest

is applicable to different quantities and types of samples rendering this method very robust and versatile [12]. Thus mutation enrichment using E-*ice*-COLD-PCR combined with Pyrosequencing for a sequence-based readout of the enriched alleles is currently the easiest, most rapid, and sensitive sequencing-based mutation detection and identification assay format.

In this chapter, we describe all steps necessary for the development of an Enhanced-*ice*-COLD-PCR assay for the detection and identification of mutations in a region of interest. We have included all steps necessary for the design of the assay, the identification of the optimal experimental conditions, and the analysis and interpretation of Pyrograms®. Results are exemplified on the analysis of *KRAS* mutations at the mutation hotspot in codons 12 and 13, which are of relevance for the choice of treatment in a variety of cancers [12].

2 Materials

2.1 Assay Design

1. Design of PCR primers using Primer 3 [13, 14] (http://bioinfo.ut.ee/primer3-0.4.0/), verification of primer efficiency (http://www.premierbiosoft.com/netprimer/) and of potential secondary structures using mFold [15] (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form).

2. Identification of polymorphisms inside primers and nonspecific primer annealing using the BLAT tool (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start).
3. Manual design of LNA blocker probe and calculation of the Tm using the Exiqon Prediction Tool (https://www.exiqon.com/ls/Pages/ExiqonTMPredictionTool.aspx).
4. Design of Pyrosequencing primers using the commercial PSQ assay design software.

2.2 DNA Extraction and Quantification

1. DNeasy® Blood and Tissue Kit.
2. QIAamp® DNA FFPE Tissue Kit.
3. Chemagic Circulating NA Kit special.
4. Quant-iT™ PicoGreen® dsDNA Assay Kit.

2.3 DNA Standards, Identification, and Preparation

1. Human genomic DNA.
2. Identification of cell lines harboring mutations of interest in COSMIC [16] (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/).
3. Genomic DNA of the cell line to be ordered at ATCC (http://www.lgcstandards-atcc.org/?geo_country=fr) or any cell line DNA repository bank.
4. NanoDrop spectrophotometer.

2.4 PCR Amplification

1. PCR primers.
2. Biotinylated PCR primers.
3. 3′ Phosphate-modified LNA blocker probes.
4. HotStar Taq DNA Polymerase.
5. dNTPs.
6. SYTO9.
7. LightCycler® 480 Multiwell Plate 96.
8. LightCycler® 96 Real-Time PCR System.

2.5 Agarose Gel Electrophoresis

1. TBE 10×.
2. UltraPure™ Agarose.
3. 100 bp DNA ladder.
4. 6× DNA Loading Dye Buffer Blue.
5. Gel molds and combs.
6. Electrophoresis apparatus.
7. Gel electrophoresis imaging system.

2.6 Sample Preparation for Pyrosequencing

1. 96-Well Low Profile PCR Plate, Skirted.
2. Streptavidin Sepharose HP beads.
3. PyroMark® Q96 Vacuum Workstation (220 V) [17].

4. PyroMark Q96 Plate Low.
5. PyroMark Q96 Sample Prep Thermoplate Low.
6. PyroMark Q96 Vacuum Prep Trough.
7. Binding buffer: 10 mM Tris–HCl, 2 M NaCl, 1 mM EDTA, 0.1 % Tween 20, pH 7.6.
8. Denaturing solution: 0.2 M NaOH.
9. Washing buffer: 10 mM Tris–acetate, pH 7.6.
10. Annealing buffer: 20 mM Tris–acetate, 2 mM Mg–acetate, pH 7.6.
11. Costar microplate sealing tape.
12. Thermomixer comfort.
13. Pyrosequencing primer.

2.7 Mutation Detection and Identification by Pyrosequencing

1. PyroMark Q96 Cartridge.
2. PyroMark Q96 MD Pyrosequencer.
3. PyroMark MD software.
4. PyroMark Gold Q96 Reagents (5 × 96).

3 Methods

The detection, identification, and quantification of mutations by Enhanced-*ice*-COLD-PCR followed by Pyrosequencing can be divided into seven steps (Fig. 2):

1. The design of the assay, which includes the design of the PCR primers, the LNA blocker probe, the Pyrosequencing primer, and the identification of cell line DNA standards.
2. The validation of the PCR primers including the identification of the best primer combination and their annealing temperature.
3. The validation of the sequencing primer and of the mutation present in the cell line used as standard for the optimization of the assay.
4. The identification of the critical temperature and the optimal concentration of the LNA blocker probe.
5. The generation of a Pyrosequencing assay allowing the identification and the quantification of all mutations of interest.
6. The DNA extraction and accurate quantification from samples of different types such as fresh frozen, FFPE, and plasma.
7. The detection and identification of the mutation by E-i*ce*-COLD-PCR followed by Pyrosequencing in the sample of interest.

3.1 DNA Extraction and Quantification from Fresh Frozen, FFPE, and Plasma Samples

For an optimal performance of E-*ice*-COLD-PCR, it is necessary that DNA extractions from different types of samples are performed properly and accurate quantification of the resulting DNA quantity has to be performed before downstream applications.

1. For fresh-frozen samples, extract genomic DNA using the DNeasy Blood and Tissue Kit according to the manufacturer's instructions.
2. For FFPE samples, extract genomic DNA using the QIAamp DNA FFPE Tissue Kit according to the manufacturer's instructions.
3. For plasma samples, extract circulating DNA using the Chemagic NA extraction kit according to the manufacturer's instructions. The volume of reagents can be adjusted according to the starting volume of available plasma.
4. Quantify genomic DNA from fresh-frozen samples using the Quant-iT™ PicoGreen® dsDNA Assay Kit according to the manufacturer's instruction.
5. Quantify DNA from FFPE and plasma samples by a quantitative real-time PCR assay using a dilution series of commercial DNA as standards (*see* **Note 1**).

3.2 Assay Design

The design of the assay is a critical step for Enhanced-*ice*-COLD-PCR and should be carefully performed to avoid any subsequent problems in the following experimental protocol. At least two pairs of PCR primers per region and two LNA blocker probes per assay should be designed and evaluated.

3.2.1 Identification of the Region of Interest and DNA Standards

1. Enhanced-*ice*-COLD-PCR is a method particularly suitable for the detection and identification of mutations in hotspot regions, where different types of mutation occur on a restricted number of nucleotides. Thus the identification of the region of interest is crucial and should include all mutations of potential interest. Identification of known mutations can be done with the help of mutation databases such as the COSMIC database listing more than one million somatic mutations throughout the genome in different types of cancer samples or cell lines.
2. The region of interest should not be larger than 200 nucleotides and its boundaries should be as much as possible free of mutations or should contain only very-low-frequency mutations. The mutation hotspots can extend from one to a few tens of codons (examples: *KRAS* codons 12 and 13, *BRAF* codon 600 and *NRAS* codon 61).
3. For the development of the assay, identify at least one cell line carrying a mutation located in the region of interest from the literature or a database such as COSMIC.

4. Order the corresponding genomic DNA directly from a cell line bank such as ATCC (*see* **Note 2**).
5. Upon receipt, determine the concentration of the cell line DNA using the Quant-iT™ PicoGreen® dsDNA Assay Kit or similar double-stranded DNA-specific quantification kits (*see* **Note 3**).
6. Genotype the genomic DNA of the different cell lines by Pyrosequencing in order to validate the presence of the correct mutations as well as their proportions (*see* Subheading 3.3).
7. Perform serial dilutions of mutant and WT commercial DNA to obtain standards with different mutation fractions, e.g., 50, 10, 5, 1, 0.5, and 0.1 % (*see* **Note 4**). Genotype the dilutions to assess the linearity and the accuracy of the dilutions (*see* Subheading 3.3).

3.2.2 Design of Amplification Primers

1. Design the PCR primers using the freely available Primer 3 software. The length of the amplification product should optimally be around 100–150 bp to be applicable to all types of samples including fragmented DNA such as cell-free circulating DNA and FFPE samples.
2. Report in brackets the mutation hotspot region of interest in order to include the target region in the proposed assays. Optimal primer size should be around 20 nucleotides and the optimal Tm around 60 °C, which are the default settings in Primer 3.
3. For all the different proposed assays, verify the absence of secondary structures using the mfold program to assess the suitability of the assays for real-time PCR. Evaluate the primer efficiencies using the online software NetPrimer.
4. For each assay, investigate the presence of potential polymorphic positions such as SNPs underlying the annealing sites as well as primer specificity using a BLAT search on the UCSC Genome Bioinformatics portal. Discard the assays where amplification primers anneal to potential polymorphic sites and/or when both primers are highly complementary to several regions of the genome (*see* **Note 5**).
5. Select at least two assays among the proposed ones.
6. Include a biotin on the 5′ end of one primer, depending on the sense of the genotyping/Pyrosequencing assay (*see* below).

3.2.3 Design of Pyrosequencing Primers

1. A Pyrosequencing primer is used to detect, identify, and quantify mutations by genotyping. Design Pyrosequencing primers using the commercial PSQ assay design software.
2. The sequencing primer should not anneal to any polymorphic and/or mutation site and should contain at least two nucleotides

not overlapping with the PCR primers on its 3′ end to avoid any unspecific signals or annealing to potential primer dimers.

3. At least the last four bases of the 3′ end of the sequencing primers should be unique in the amplification product to avoid the generation of background signals, which could interfere with accurate quantification.
4. If possible, select the sense of the sequencing reaction to avoid the presence of "T" mutations, as the complementary Pyrosequencing reaction will incorporate an "A" nucleotide. Adenosine triphosphates can be used as substrate by the luciferase despite the use of dATPαS and can induce a high background signal [18].
5. Select the sense of the sequencing reaction according to the ease of interpretation of the Pyrograms in one direction or the other around the mutation hotspot.
6. Do not place the Pyrosequencing primer too far from the mutation hotspot region as the PyroMark Gold Q96 Reagents used for genotyping experiments can only sequence 30–40 nucleotides.

3.2.4 Design of Pyrosequencing Assays

Pyrosequencing assays are designed using the PyroMark MS software supplied with the Pyrosequencer for genotyping experiments.

1. To design a genotyping assay, insert the DNA sequence beginning at the base beyond the 3′ end of the sequencing primer into the text box "sequence to analyze" in the "simplex entries" tab. The sequence must cover the mutation hotspot region and also include nucleotides, which are known to be not mutated and are used as control nucleotides.
2. Include at least one mutation in the nucleotide sequence using the IUPAC nomenclature for the polymorphism. The "dispensation order" tool generates the order in which the different nucleotides are dispensed.
3. For the development of E-*ice*-COLD-PCR assays and the setup of the experimental conditions, cell line DNA standards bearing known mutations are used. For these steps, create a genotyping assay including only the one mutation specific for the cell line, which is easily manageable by the software. It allows accurate quantification of the mutation.
4. For the detection, identification, and quantification of unknown mutation in the region of interest, insert the nucleotide sequence for all possible combinations of mutations in a single genotyping assay.
5. The software will indicate in the warnings window that the sequence might be too complicated or is ambiguous to allow

an automated determination or quantification of all possible mutations. If the software indicates that "AQ mode cannot be performed" or that "some genotypes will generate the same sequence patterns and will not be distinguishable," the input sequence should be simplified. In this case, leave only the most frequent mutations in the sequence to generate the dispensation order, which can be quantified by the software.

6. Add manually to the dispensation sequence the remaining possible mutations, which need to be included for mutation screening. These nucleotides are considered by the software as control nucleotides generating no peaks. If these mutations are present in a sample, the software considers it as a failed sample because the dispensation pattern is uncertain. These samples need to be distinguished from failed samples with no signal.
7. Analyze manually the Pyrograms of "failed samples" to detect and identify the presence of possible mutations. Use raw peak intensities for manual or automated mutation quantification (*see* **Note 6**).

3.2.5 Design of LNA Blocker Probe

1. The LNA blocker probe inhibits elongation of one WT DNA strand during PCR as its nucleotide sequence is fully complementary to the WT sequence. Design the LNA blocker probe over the possible mutated nucleotides between the two amplification primers and overlap five to six nucleotides with the 3′ end of one of the amplification primers.
2. The primer partly complementary to the blocker probe should be the most stable one in order to avoid the formation of primer dimers and/or primer self-dimers. Determine the most stable primer bioinformatically using the NetPrimer software.
3. If possible, center the LNA blocker probe on the mutation hotspot and include some nucleotides, which are known to be not mutated.
4. LNA bases allow increased single-nucleotide discrimination by maximizing the Tm differences between a single-nucleotide mismatch compared to a perfect match, except for G-T mismatches when the G is present on the DNA strand bearing LNA bases [19]. Select the sense (forward or reverse) of the LNA blocker probe to include as few as possible G nucleotides in the region containing the mutation.
5. Preferentially include LNA bases on each nucleotide likely to be mutated in order to generate mismatches. If the potentially mutated sequences include only one nucleotide, include an LNA triplet in the probe centered on the nucleotide of interest. Up to six consecutive LNA bases can be included in the blocker probe if the mutation hotspots are composed of less than ten consecutive nucleotides. If the potentially mutated

nucleotides are spread over several tens of nucleotides, include one LNA base every two, three, or four nucleotides in the blocker probe.

6. At least six nucleotides at the 5′ and 3′ ends of the blocker probe should be devoid of LNAs.
7. Verify the Tm of the LNA blocker probe bioinformatically using the Exiqon Tm Prediction Tool. The Tm should be comprised between 75 °C and 90 °C. If not, modulate the length of the LNA blocker probe and/or the number of incorporated LNA bases.
8. Add a phosphate residue to the 3′ end of the probe to avoid any elongation of the LNA blocker probe during the PCR reaction.

3.3 Determination of the Optimal E-ice-COLD-PCR Conditions

For each region of interest at least two PCR primer pairs should be ordered for evaluation and only the very best ones should be selected for further Enhanced-*ice*-COLD-PCR application.

3.3.1 Validation of PCR Primers

1. The first step of the validation consists of the determination of the optimal annealing temperature for each primer pair. This is performed on a gradient thermocycler using 25 ng of commercial WT genomic DNA as template.
2. Typical PCR reaction conditions are 1× HotStar Taq buffer supplemented with 1.6 mM $MgCl_2$, 200 mM of each dNTP, 1.0 U of HotStar Taq polymerase, and 200 nM of forward and reverse primers in a total volume of 25 μL.
3. Typical PCR cycling conditions include an initial denaturation step performed 10 min at 95 °C, followed by 50 cycles of 30-s denaturation at 95 °C, 20-s of a gradient annealing ranging from 50 to 70 °C, and 10-s elongation at 72 °C. Final extension is performed 5 min at 72 °C and the cooling is performed at 40 °C.
4. Deposit 10 μL of PCR product supplemented with 1× loading dye as well as a DNA ladder sample on a 2 % agarose gel including a fluorescent dye (example: ethidium bromide). Perform a horizontal electrophoresis in TBE 1× at 5 V/cm during 1 h. The specificity of the amplifications is assessed by evaluation of the length of the PCR product and the highest temperature allowing a strong and specific amplification product is selected as the optimal annealing temperature.
5. Evaluate the different PCR primer pairs for a real-time PCR application using the optimal annealing temperature. PCR reaction conditions are the same as described in **step 2** supplemented with 2 μM of SYTO9 as fluorescent dye.
6. PCR cycling conditions include an initial denaturation step for 10 min at 95 °C, followed by 50 cycles of 30-s denaturation at

95 °C, 20 s at the optimal annealing temperature, and 10-s elongation at 72 °C. Acquire fluorescence at the end of the annealing step in each cycle. Final steps include a melting curve performed from 65 °C to 97 °C and cooling to 40 °C (*see* **Note 7**).

7. Amplification curves of each assay allow the identification of nonspecific products if some signal is present in the no-template reactions. Melting curves allow the determination of the Tm of the PCR amplification product and of primer dimers present in the no-template reactions. Select the assays generating no or late fluorescent signal in the no-template reaction for further application of the E-*ice*-COLD-PCR assays.

3.3.2 Determination of the Critical Temperature, the Optimal Blocker Probe Concentration, and the Limit of Detection of the Assay

The critical temperature (Tc) is the optimal temperature where blocker-mutant heteroduplexes are denatured while blocker-WT homoduplexes remain hybridized. This temperature is essential in E-*ice*-COLD-PCR reaction as it allows an optimal enrichment of the mutation. Figure 3 shows an example of the optimization of the critical temperature using different temperatures and blocker concentrations.

1. Prepare a stock solution for LNA blocker probes at a concentration of 10 μM. Prepare a 250 nM dilution from the stock solution. Store all solutions at −20 °C.
2. To determine the critical temperature, perform an E-i*ce*-COLD-PCR using a Tc gradient, different concentrations of LNA blocker probes, and 25 ng of a DNA standard sample containing a mutation fraction of 1 or 0.5 %. Recommended concentrations for the blocker probes are as follows: 0, 10, 20, 30, and 50 nM (Fig. 3).
3. PCR reaction conditions are 1× HotStar Taq buffer supplemented with 1.6 mM $MgCl_2$, 200 mM of each dNTP, 1.0 U of HotStar Taq polymerase, 200 nM of forward and reverse primers, 2 μM of SYTO9, and different concentrations of blocker probes in a 25 μL volume.
4. PCR cycling conditions include an initial denaturation step for 10 min at 95 °C, followed by six cycles of standard PCR (30-s denaturation at 95 °C, 20 s at optimal primer annealing temperature, and 10-s elongation at 72 °C) and 44 cycles of E-i*ce*-COLD-PCR (20-s denaturation at 95 °C, 30-s blocker annealing at 70 °C, 20 s at Tc using a temperature gradient ranging from 70 °C to 90 °C, 20 s at optimal primer annealing temperature, and 10-s elongation at 72 °C). Acquire fluorescence at the end of the annealing step in each cycle. Final steps include a melting curve performed from 65 °C to 97 °C and cooling to 40 °C (*see* **Note 8**).

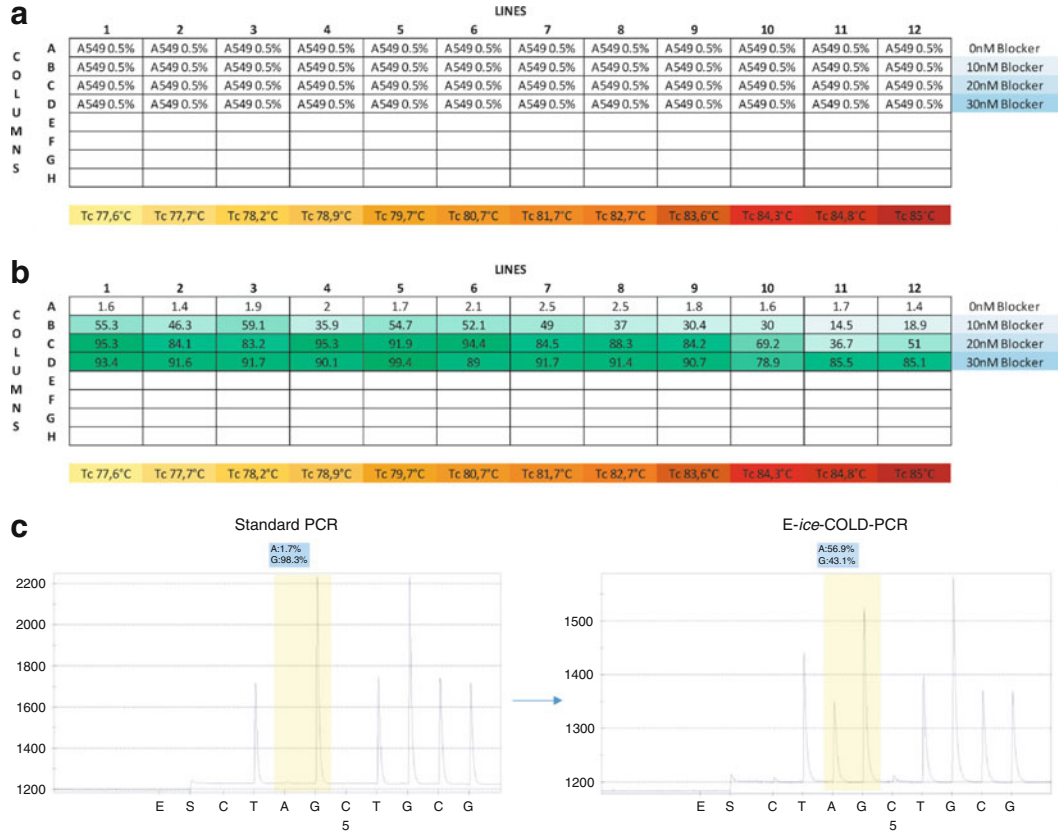

Fig. 3 Example of the determination of the critical temperature for the E-*ice*-COLD-PCR analyzing the mutation hotspot in *KRAS* codons 12 and 13 and using the 114 bp assay and the 31 bp LNA blocker published in [12]. (**a**) Example of a plate layout using a *KRAS* c.34G>A mutated cell line (A459) diluted to 0.5 % mutated allele, different blocker concentrations from 0 to 30 nM, and a Tc gradient ranging from 77.6 °C to 85 °C. (**b**) Quantification of the mutation enrichment obtained after Pyrosequencing for the plate layout presented in (**a**). The selected Tc is 80 °C. (**c**) Example of Pyrograms obtained for a 0.5 % mutation fraction of the *KRAS* c.34G>A mutation (A549) after standard and E-*ice*-COLD-PCR

5. Monitor the effect of the blocker probe on the amplification plots, which are with increasing blocker concentration progressively delayed compared to amplifications without blocker probes. For some Tc and/or concentrations of the blocker probe, the inhibition effect could be very strong resulting in the absence of PCR amplification products and/or in the presence of a large amount of primer dimers.
6. Analyze 10 μL of all PCR amplification products by Pyrosequencing to quantify the enrichment of the mutations (*see* Subheading 3.4).
7. In the resulting Pyrogram (output format of the Pyrosequencer), the enrichment of the mutation should be

clearly visible for a large range of temperatures and correlate positively with increasing blocker probe concentration. For each blocker probe concentration the optimal Tc should be the temperature where the highest enrichment is achieved together with an excellent quality of the Pyrograms, i.e., single-nucleotide peak intensity higher than 100 units.

8. Test the performance of the E-*ice*-COLD-PCR assay using the same PCR conditions (**steps 3** and **4** above) at the selected Tc using the different blocker probe concentrations (prepared in Subheading 3.3.2, **step 2**) including new concentrations and excluding non-informative concentrations (*see* **Note 9**). Perform the experiments with the dilutions of the cell line DNA of 1 % and lower dilutions (0.5, 0.1, 0.05, and 0.01 %) (*see* **step 4** in Subheading 3.2.1) and WT commercial samples in order to determine the limit of detection of the assay and the optimal blocker probe concentration.
9. Perform **steps 5** and **6** on these amplifications.
10. Identify the optimal blocker probe concentration, which corresponds to the concentration where the highest mutation enrichment is achieved for the lowest mutation fraction associated to a high quality of the resulting Pyrogram and where the WT sample shows a mutation value close to 0 % (*see* **Note 10**).
11. Validate the optimal conditions of the assay using the determined Tc and blocker probe concentration on the whole dilution series of the cell line including also WT and no-template samples.
12. The optimal concentration of the blocker probe depends on the quality and the quantity of DNA present in the analyzed samples and must therefore be adapted to the starting amount of DNA. DNA from FFPE and plasma samples is degraded and this should be taken into account for the choice of LNA blocker probe concentration. Moreover, when the starting amount of DNA decreases, the LNA blocker probe concentration can be decreased simultaneously as the lower number of molecules present in the reaction requires a lower sensitivity. Use the determined concentration of blocker probe for DNA of high quality such as fresh-frozen sample. For DNA from FFPE or circulating DNA, reduce the concentration of the blocker probe by 10–20 nM.
13. Once optimal PCR primer annealing temperature, critical temperature, and concentration of LNA blocker probes have been identified, analyze the different samples of interest by E-*ice*-COLD-PCR assays preferentially in duplicate or triplicate experiments.

3.4 Mutation Detection, Identification, and Quantification by Pyrosequencing

This step allows the preparation of the PCR products for Pyrosequencing experiments. It includes the purification and the denaturation of the amplification product, which will be rendered single stranded, and the hybridization of the sequencing primer.

3.4.1 Sample Preparation

1. Transfer 10 μL of E-*ice*-COLD-PCR product into a standard skirted 96-well PCR plate and add 40 μL of binding buffer, 2 μL of Sepharose beads, and 28 μL of water. Seal the plate and incubate for 10 min at room temperature under constant mixing (1,400 rpm), which is required for the capture of the biotinylated PCR products by the Sepharose beads.
2. In parallel, prepare the Pyrosequencing plate by diluting 4 pmol of the Pyrosequencing primer into 12 μL of annealing buffer into the respective wells of the PSQ plate. Different Pyrosequencing primers can be used in different wells of the same plate.
3. Fill the four troughs of the vacuum preparation tool with 100 mL of 70 % ethanol, 100 mL 0.2 M NaOH denaturing solution, 120 mL of washing buffer, and 150 mL of water. More washing buffer should be put in the troughs compared to ethanol and NaOH to ensure the complete removal of NaOH, which might inhibit the downstream Pyrosequencing reactions.
4. Turn on the workstation to create a vacuum in the aspiration device (450 mmHg). Immerse the tips in water for several seconds for cleaning. Then, the PCR plate can be removed from the mixer and the binding mix can be aspirated. The beads remain on the filters of the tips.
5. After all binding mix has been aspirated, immerse the tips in ethanol 70 %, NaOH denaturing solution, and washing solution for 5 s, 5 s, and 10 s, respectively. Let the vacuum dry the beads for 10 s and turn it off above the PSQ plate.
6. Next, the filter tips can be immersed in the annealing mix of the PSQ plate in order to release the beads into the wells.
7. Heat the sequencing plate for 2 min at 80 °C on a thermoplate placed on a heating device for single DNA strand denaturation. Cool the plate at room temperature for 5 min to allow annealing of the sequencing primer.

3.4.2 Pyrosequencing Reaction

1. Create a new Pyrosequencing run on the Pyrosequencer using the PyroMark MD software. Indicate the genotyping assay used in the selected wells and the name and/or conditions of each wells.
2. Fill the tips of the cartridge with the corresponding reagents and enzyme mix and dNTPs avoiding bubble formation. 180 μL of reagents and enzyme mix are sufficient for a full

plate and at least 150 μL of each dNTPs should be used. dNTPs are not limited in the kit and can be used in excess (*see* **Note 11**).

3. Deposit a sealed Pyrosequencing test plate and the reagents' cartridge in the Pyrosequencer and perform the dispensation test to verify if the dispensing tips are working properly. Droplets should be clearly visible and homogeneous and the tips should be changed if necessary.
4. Remove the test plate, put the cooled prepared Pyrosequencing plate, and start the run. The length of a run is proportional to the number of dispensations (1/min) and a genotyping run should not exceed 30 min.
5. After the end of the sequencing run, rerun a dispensation test to verify if some tips became blocked during the run. If the tips were blocked or if no signal was present because of exhausted or degraded reagents, the Pyrosequencing plate should be resuspended in 20 μL of binding buffer, and re-purified by the protocol described in **step 2** in Subheading 3.4.1.
6. Analyze the results with the PyroMark MD software in AQ mode, which allows accurate quantification of the mutation(s) of interest. For more complicated patterns and mutation hotspot regions, the Pyrograms need to be manually analyzed for identification of the mutation and the intensity peaks have to be manually processed or with a help of a "homemade" program for mutation quantification (*see* Subheading 3.2.4).
7. Export the results in .txt format to enable further treatment and analysis with statistical or graphical software such as Excel®.
8. Remove the cartridge and clean the tips with distillated water. Remaining reagents can be preserved at 4 °C and reused while dNTPs can be discarded.

An example of Pyrograms before and after enrichment of *KRAS* mutation in colorectal cancer samples is shown in Fig. 3c.

4 Notes

1. The best performing assay designed for the E-*ice*-COLD-PCR application in the region of interest should be used for the quantitative PCR omitting of course the blocker probes used for the selective amplification of mutated alleles. The quantity of amplifiable DNA of the region of interest can be accurately determined using a dilution series of commercial genomic DNA ranging from 25 ng to 25 pg as calibration standard. Typical quantitative PCR reaction conditions are 1× HotStar Taq buffer supplemented with 1.6 mM $MgCl_2$, 200 mM of

each dNTP, 1.0 U of HotStar Taq polymerase, 2 μM of SYTO9, and 200 nM of forward and reverse primers in a total volume of 25 μL. Typical quantitative PCR cycling conditions include an initial denaturation step performed 10 min at 95 °C, followed by 55 cycles of 30-s denaturation at 95 °C, 20 s at annealing temperature, and 10-s elongation at 72 °C. Fluorescence acquisition is performed during each cycle at the end of the primer annealing step. Final steps include a melting curve performed from 65 °C to 97 °C and cooling to 40 °C.

2. If no cell lines with a mutation in the region of interest can be identified, a validated sample presenting a mutation in the region of interest could be used instead. Thus, whole-genome amplification (WGA) should be performed on the sample using REPLI-g Midi kit (Qiagen) according to the manufacturer's instructions and mixed with a WT WG amplified sample to obtain the different mutation fraction dilution. It is not recommended to mix a WGA mutant sample with an unamplified WT sample, as due to the different DNA fragment sizes, differences in the amplification efficiency might occur.
3. Quantification of a DNA sample based on the measurement of the absorption/optical density only is not sufficiently accurate and must be complemented by more precise quantification methods such as intercalating dyes or quantitative real-time PCR.
4. The dilutions series allows the determination of the limit of detection of each E-*ice*-COLD-PCR assay.
5. Some regions can be extremely homologous to the region of interest such as pseudogenes, for example the *KRAS* pseudogene 1 (*KRASP1*), and it is primordial to have at least five nucleotides specific only to the region of interest on the 3′ end of at least one primer.
6. Microsoft Excel Visual Basic Applications (VBA) are a good platform for the development of an automated treatment of raw peak intensities for mutation detection, identification, and quantification. An example of a developed MS VBA for the analysis of the *KRAS* codon 12 and 13 mutation hotspot is shown in [12].
7. The melting curve analysis can also be performed as high-resolution melting (HRM) analysis, which can also be used for mutation detection at the end of the E-*ice*-COLD-PCR prior to downstream experiments. At least 20 acquisitions per degree should be included in the melting program to achieve a satisfying analytic resolution.
8. The use of a gradient real-time thermocycler such as LightCycler® 96 Real-Time PCR system can significantly improve the setup efficiency of E-*ice*-COLD-PCR assays as amplification plots and primer dimer formation can be monitored

directly during the PCR, before Pyrosequencing experiments. If no gradient real-time thermocycler is available, a standard gradient thermocycler can be used for the optimization steps.

9. If the first experiments for the determination of the Tc show no mutation enrichment for the lowest tested blocker concentration, these concentrations should be excluded from further analysis while if the quality of the Pyrogram remains high for the highest tested concentration, higher concentrations could and should be tested in the following experiments.
10. The limit of detection corresponds to the lowest mutation fraction where mutation enrichment is still visible compared to the WT sample. As Pyrosequencing presents a resolution of 5–10 %, a mutation difference of at least 10 % between WT and mutated samples after E-*ice*-COLD-PCR should be taken as cutoff to maximize the sensitivity and the specificity of the E-*ice*-COLD-PCR assay. After E-*ice*-COLD-PCR, a WT sample can present with a higher mutation background compared to a standard PCR. This is due to the stringency/aggressiveness of the method and the blocker probe concentration should be optimized to minimize this effect.
11. Reaction volumes and required input of the amplification product might vary depending on the model of the Pyrosequencing machine.

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