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Expert Review of Molecular Diagnostics

ISSN: 1473-7159 (Print) 1744-8352 (Online) Journal homepage: http://www.tandfonline.com/loi/iero20

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To cite this article: Jörg Tost (2015): The clinical potential of Enhanced-ice-COLD-PCR, Expert Review of Molecular Diagnostics, DOI: 10.1586/14737159.2016.1123623

To link to this article: http://dx.doi.org/10.1586/14737159.2016.1123623

Accepted author version posted online: 20 Nov 2015.
Published online: 15 Dec 2015.

EXPERT REVIEW OF MOLECULAR DIAGNOSTICS, 2015
http://dx.doi.org/10.1586/14737159.2016.1123623

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EDITORIAL

The clinical potential of Enhanced-*ice*-COLD-PCR

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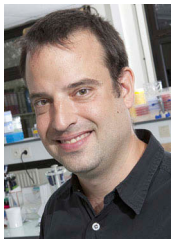
ABSTRACT

Enhanced-*ice*-COLD-PCR (E-*ice*-COLD-PCR) is a novel assay format that allows for the efficient enrichment and sensitive detection of all mutations in a region of interest using a chemically modified blocking oligonucleotide, which impedes the amplification of wild-type sequences. The assay is compatible with DNA extracted from tissue and cell-free circulating DNA. The main features of E-*ice*-COLD-PCR are the simplicity of the setup and the optimization of the assay, the use of standard laboratory equipment and the very short time to results (~4 h including DNA extraction, enrichment and sequence-based identification of mutations). E-*ice*-COLD-PCR is therefore a highly promising technology for a number of basic research as well as clinical applications including detection of clinically relevant mutated subclones and monitoring of treatment response or disease recurrence.

KEYWORDS

Cell-free circulating DNA; COLD-PCR; E-*ice*-COLD-PCR; mutation; *BRAF*; *KRAS*; *NRAS*; personalized medicine; pyrosequencing



There is currently great interest in mutation detection for the genotype-driven personalized treatment of cancer patients. Low amounts of tumor-derived DNA molecules can be found in various body fluids such as urine or sputum or as cell-free circulating DNA molecules that can be isolated from the serum/plasma of patients.[1–3] The analysis of cell-free DNA bears great promise for the early detection of disease, monitoring of tumor burden and response to treatment as well as the screening for disease recurrence and development of treatment resistance. Furthermore, tumors might present with a large degree of heterogeneity with clinical important mutations being only present in a subset of the tumor cells and tissue samples might contain a large amount of nontumoral cells. It has, therefore, 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. However, while methods based on allele-specific PCR primers or real-time PCR probes, which are currently predominantly used in commercially available "research use only" or companion diagnostic assays, do show the required sensitivity, they do require prior knowledge of the mutation or a number of assays have to be performed in parallel to test each known mutation. Furthermore, they might lead to false-positive and false-negative results if spurious amplification occurs during early PCR cycles due to suboptimal amplification conditions, mutations involving more than one nucleotide base are present or other unknown mutations are present. These problems are resolved in assays using sequencing-based mutation detection, which enable the identification of the mutated nucleotide and do not require prior knowledge of the mutation. Standard Sanger and next-generation sequencing (NGS) approaches do, however, not provide the required analytical sensitivity. To avoid confounding by sequencing errors, specialized NGS protocols utilizing molecular barcodes unique for each template molecule, such as Safe-SeqS,[4,5] or the sequencing of circularized barcoded DNA as used in the CypherSeq approach [6] have been developed and allow the detection of very rare mutations. However, requiring a large amount of sequencing to detect rare mutations, these methods are relatively costly and time-consuming, allowing only a limited number of samples to be processed in parallel. They are equaled in their sensitivity by a large variety of PCR-based enrichment methods that have been developed to enrich for unknown mutations in specific loci of interest. These strategies include, notably, several approaches based on co-amplification at lower denaturation temperature (COLD) PCR including *full-* and *fast-*COLD PCR.[7–9] However, mutation enrichment using these approaches is not efficient for all types of mutations; a problem that was overcome with the development of an assay termed "improved and complete enrichment (*ice*)-COLD-PCR."[10] *ice*-COLD-PCR introduces a

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nonextendable blocker probe that is complementary to the wild-type (WT) sequence and that overlaps with five nucleotides at the 3′ end of each primer, which is used to block WT-homo- and WT-mutant heteroduplex formation.[10] Identification of the mutations can be performed using Sanger, pyrosequencing or targeted amplicon sequencing on NGS instruments.[10,11] While being a major improvement, *ice*-COLD-PCR, like other COLD-PCR-based methods, requires a critical temperature (*Tc*) for the selective denaturation of the mutant blocker oligonucleotides, which has to be very accurate as a slight variation of as little as 0.2°C can completely abolish the mutation enrichment.[10] Very few real-time thermocyclers do allow for such a precise definition of the temperature. Moreover, the *ice*-COLD-PCR requires a significant amount of work to determine the correct *T*c and the *ice*-COLD-PCR is performed as a nested PCR on a standard PCR amplification product.[10] This first amplification step could possibly lead to the loss of rare mutations in the amplification product. Therefore, we developed Enhanced-*ice*-COLD-PCR (E-*ice*-COLD-PCR) as a modified version of the *ice*-COLD-PCR, which contains chemically modified nucleotides (locked nucleic acid [LNA] bases) within the blocker probe.[12] The main difference to the original protocol is the high specificity of LNA-containing probes, which are also used for highly selective and specific amplification in challenging applications such as the quantitative PCR (qPCR) -based assays for the differentiation of miRNAs and isoMirs (Exiqon, Vedbaeck, Denmark) or the SMART-seq V4 kits for library preparation from ultra-low input samples or single cells (Clontech Inc., Mountain View, CA, USA). The incorporation of LNAs in the blocker oligonucleotides enables a more efficient enrichment of mutations over a large range of temperatures making the method easily usable on different thermocycler platforms compared to the original approach and the need for a preamplification step is abolished.[12] E-*ice*-COLD-PCR can be applied to different quantities and types of samples, including DNA extracted from fresh frozen, formalin-fixed paraffin-embedded samples and most importantly DNA obtained through noninvasive procedures such as liquid biopsies, rendering this method very robust and versatile.[12,13] It should be pointed out that the number of DNA molecules present after extraction and the subsequent quantity of input DNA into the E-*ice*-COLD-PCR reaction will determine the possible limit of detection.[13] To confirm the identity of the enriched mutations, we do routinely sequence the amplification product by pyrosequencing a quantitative high-resolution sequencing-by-synthesis technology using dedicated benchtop instruments. While the use of pyrosequencing has the non-negligible advantage of delivering the sequence identity with high precision in a very short time span (~10 min for the dispensation of 10 nucleotides enabling the analysis of a typical mutation hot spot), other read-out technologies such as conventional Sanger sequencing or next-generation sequencing instruments could also be used. Nonetheless, it should be pointed out that Sanger sequencing has a lower quantitative resolution, which could be problematic if a very rare mutation (e.g., <0.5%) has only been partly enriched. NGS instruments will require parallel analysis of a large number of samples to be cost-effective as after enrichment a small number of reads will be sufficient to reliably detect the enriched mutations.[11] E-*ice*-COLD-PCR assays are performed in a convenient 96-well plate format compatible with most standard laboratory equipment such as real-time thermocyclers and (pyro)sequencers. The main features of the optimized E-*ice*-COLD-PCR protocol are the relative simplicity of the setup and optimization of the assay and the short time to results, which allows its implementation for a number of basic research as well as clinical applications. Including the DNA extraction step from (formalin-fixed paraffin-embedded) tissue or plasma,[14] the enrichment PCR as well as the mutation identification by pyrosequencing results can be obtained within 4 h, which makes the workflow highly compatible with the management of patients and allows for a rapid decision on the suitability of a specific treatment. A detailed protocol on how to set up, optimize and validate E-*ice*-COLD-PCR assays has recently been published.[15] Published optimized assays from our laboratory include the analysis of mutations in *KRAS, BRAF* and *NRAS*.[12,13,15–17] Although the enrichment step does not allow for an exact quantification of the mutation level, the simultaneous analysis of samples with a known mutation level, such as cell lines diluted into WT DNA, enables a semiquantitative assessment of the mutation level in the original sample.[13] Of note, the same assay format, that is, using LNA-modified blocker probes in an *ice*-COLD-PCR reaction, was developed in parallel to our efforts by Transgenomic Inc. (Omaha, NE, USA). Multiplex assays enriching mutations in several mutation hot spots in *BRAF, KRAS, NRAS, PIK3CA* and EGFR are currently commercially available (ICEme™ MX-ICP Mutation Enrichment Kits, Transgenomic Inc., Omaha, NE, USA). The commercially proposed enrichment protocol allows for the multiplex enrichment of several regions of interest and is compatible with a number of read-out technologies, including Sanger and NGS-based sequencing or qPCR-based assays.

E-*ice*-COLD-PCR has received considerable interest as it has several applications for basic research and clinical questions.

Detection of low levels of mutations in clinical tissue samples

E-*ice*-COLD-PCR allows detecting subclones in a tumor sample that might have an impact on the clinical management of patients and that might not easily be detected by conventional technologies. For example, it has been shown using the commercial *ice*-COLD assays that mutations in exon 2 of *NRAS* down to a mutation level of 0.5% did negatively impact survival to a similar extent as patients with high levels of *NRAS* mutations.[18] This clinically relevant threshold is difficult to measure using most standard mutation detection technologies, while it can be detected with high confidence using (E)-*ice*-COLD-PCR assays. In the same line of applications, we have been able to show that most congenital melanocytic nevi carry a *NRAS* mutation, that this is the sole recurrent mutation and its presence in specific subpopulations is probably driving the melanocytic proliferation in this type of nevi.[16,17] E-*ice*-COLD-PCR was able to detect *NRAS* mutations in samples, which were tested negative by exome sequencing, Sanger and standard pyrosequencing as well as high-resolution melting analysis.[16] However, very sensitive detection of mutations without quantitative information might also be problematic in some instances. For colorectal cancer patients, overall and progression-free survival differs in function of the presence of a *KRAS* mutation down to a mutation level of 1% when treated with an anti-EGFR therapy requiring, thus, a sensitive detection of the mutation.[19] Lower levels of mutations did, however, not significantly negatively impact the survival compared to patients with *KRAS* WT tumors. Analyses using enrichment methods would need to be very carefully controlled using standards with a known level of mutations analyzed in parallel, allowing to categorize samples in groups with mutation levels of, for example, >5%, between 1 and 5%, and <1%. For clinical decisions on patients with a mutation level in the order of the clinically important threshold, E-*ice*-COLD-PCR will probably need to be complemented with quantitative technologies such as digital PCR. Nonetheless, it will, as a prescreening method, enable to focus these complementary analyses on a subset of the samples. The same is probably true when subtle changes in the ratio of two mutations might have an impact on treatment success as it was reported for the ratio of the T790M/activating *EGFR* mutations in the development of resistance to a third-generation EGFR inhibitor in lung cancer.[20,21]

Monitoring disease evolution during treatment

Monitoring the presence and quantitative evolution of the level of mutations in cell-free DNA has been proposed as a tool to assess the efficacy of a treatment. Similar to using the total quantity of cell-free DNA as a pharmacodynamic marker for tumor evolution,[22] the level of specific mutations can be followed during treatment using technologies such as the FACS-based BEAMing (beads, emulsification, amplification and magnetics) method, digital PCR or high-resolution melting–COLD-PCR.[23–25] Of specific interest, changes in the mutation level often precede the changes in the clinical response, providing a window of opportunity for retailoring the treatment regimen at an earlier timepoint. Although currently no studies have been published using E-*ice*-COLD-PCR for this purpose, this application is ideally suited for E-*ice*-COLD-PCR as results are available a few hours after a liquid biopsy, allowing a truly real-time monitoring of the treatment efficacy.

The development of multiplexed E-*ice*-COLD-PCR assays combined with NGS-based read-out on benchtop sequencers such as Illumina's Mi-seq or Ion Torrent's PGM will allow analyzing a greater number of targets at the same time, and might be particularly important if only a very limited amount of DNA is available. Furthermore, as previously shown for COLD and *ice*-COLD-PCR,[11] few reads are sufficient to reliably detect mutations after enrichment, thus allowing the analysis of a larger number of samples at lower cost in a sequencing run. The current workflow using mutation enrichment through E-*ice*-COLD-PCR combined with the pyrosequencing-based read-out has, however, a shorter time to results and does not require any sophisticated bioinformatics. Speed is also an advantage in comparison to digital PCR assays that provide similar information on the presence of mutations, but with the additional advantage of yielding quantitative results. If accurate quantification is required, E-*ice*-COLD-PCR will allow focusing these analyses on a subset of the samples with mutation levels close to the clinically important threshold.

In summary, if no highly accurate quantification is required, mutation enrichment using E-*ice*-COLD-PCR combined with pyrosequencing is currently one of the most simple, rapid and sensitive assay formats for the sequencing-based detection and identification of mutations that will be of great use for the accurate analysis of clinical samples.

Financial & competing interests disclosure

Work in the laboratory of J Tost is supported by grants from the ANR (ANR-13-EPIG-0003-05 and ANR-13-CESA-0011-05), Aviesan/INSERM (EPIG201401 and EPIG2014-18), INCa (PRT-K14-049), the joint CEA-EDF-IRSN program (CP-PHE-102) and the institutional budget of the Centre National de Génotypage (Evry, France). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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