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DNA melting analysis: Application of the "open tube" format for detection of mutant *KRAS*

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ABSTRACT

High-resolution melting (HRM) analysis is a very effective method for genotyping and mutation scanning that is usually performed just after PCR amplification (the "closed tube" format). Though simple and convenient, the closed tube format makes the HRM dependent on the PCR mix, not generally optimal for DNA melting analysis. Here, the "open tube" format, namely the post-PCR optimization procedure (amplicon shortening and solution chemistry modification), is proposed. As a result, mutation scanning of short amplicons becomes feasible on a standard real-time PCR instrument (not primarily designed for HRM) using SYBR Green I. This approach has allowed us to considerably enhance the sensitivity of detecting mutant *KRAS* using both low- and high-resolution systems (the Bio-Rad iQ5–SYBR Green I and Bio-Rad CFX96–EvaGreen, respectively). The open tube format, though more laborious than the closed tube one, can be used in situations when maximal sensitivity of the method is needed. It also permits standardization of DNA melting experiments and the introduction of instruments of a "lower level" into the range of those suitable for mutation scanning.

High-resolution melting (HRM)[1] analysis is currently one of the most effective methods for revealing gene polymorphism and mutations [1–5]. Its theoretical basis is the dependence of the melting temperature (T_m) of DNA on its length, GC content, nucleotide sequence, and degree of strand complementarity [6]. Thanks to the recent appearance of the "saturating" fluorescent DNA dyes, next-generation instruments that collect fluorescence data at higher temperature resolution, and sophisticated software, it has become possible to identify single nucleotide polymorphisms in long (up to 1 kb) DNA sequences [5,7–10].

A very important application of HRM is also mutation scanning of hot spots, i.e., the short stretches of DNA where variations appear most often [1,5,8,11–13]. The presence of mismatched bases in DNA heteroduplexes is judged from typical changes in the melting curves. This method is sensitive enough to detect a small proportion (~5%) of mutant DNA against a background of wild-type sequences [1,14]. An important advantage of HRM analysis is the "closed tube" format, which is simple, rapid, high throughput, and excludes sample cross-contaminations.

There are, however, some things that should be taken into the account when assessing this method. One of these is the high cost of HRM equipment. In addition, the closed tube format, though simple, convenient, and efficient, makes the HRM analysis dependent on the composition of the PCR buffer. Therefore, HRM, being highly sensitive to solution chemistry [15], may be performed under conditions far from optimal. For instance, the post-PCR addition of 1.0 M KCl and 0.5 M Tris–HCl (pH 8) resulted in increased HRM sensitivity and specificity [1,16]. The same amplicon is often produced in different PCR buffers (whose composition in commercial PCR kits is often unknown), thereby posing problems in standardization of HRM analyses.

It is noteworthy also that PCR mixes contain the Tris buffer whose pH is temperature dependent (at the moment of strand separation the pH is appreciably lower than at room temperature). This circumstance is not always taken into account but may influence the melting curves. Finally, the closed tube format allows DNA melting under conditions of potentially active (ongoing) DNA synthesis, for which all the required ingredients are present (the dNTPs, primers, templates, polymerase, and salts) and which can interfere with free recombination of DNA strands and formation of heteroduplexes. The contribution of nascent and, probably, heterogeneous DNA chains into the total melting curve is also unknown.

In this study, we attempted to make mutation scanning of short amplicons feasible for a standard real-time PCR instrument (not primarily designed for the HRM) and SYBR Green I dye. With this aim we used the "open tube" format to make the DNA melting independent of PCR conditions. As a model system, we chose the *KRAS* (GenBank Accession Number NG_007524) mutations in

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[1] *Abbreviations used:* DMA, DNA melting analysis; HRM, high-resolution melting; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

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exon 2 (codons 12 and 13) frequently found in many cancers. Detection of such mutations is very important because it helps determine the prognosis and treatment strategy for patients with colorectal and non-small-cell lung cancer [14,16,17]. Cost-effective methods are highly needed for implementation of such analyses in clinical laboratories.

The post-PCR optimization of DNA melting analysis (DMA) conditions (amplicon shortening and solution chemistry modification) has allowed us to considerably enhance the sensitivity of detecting mutant *KRAS* against a strong background of wild-type sequences for both low- and high-resolution DNA melting analyses.

Materials and methods

DNA samples

DNAs from various sources (blood cells, cultured cells, and tumor specimens) were isolated by phenol–chloroform deproteinization. Fluorimetric measurements of DNA samples stained with SYBR Green I in standard 96-well plates were made with the use of a Plate Chameleon V multilabel counter (Hidex Oy, Turku, Finland). Blood samples (5 ml) from healthy donors and samples of colorectal cancer after surgical resection were obtained from the Cancer Research Center (Moscow, Russia) with informed consent. The human colon carcinoma cell line SW480, which has lost the normal allele of *KRAS* and contains only the allele with mutated codon 12 (GGT → GTT) [18], was kindly provided by Dr. M. Yakubovskaya (Institute of Carcinogenesis, Moscow, Russia). Different ratios of alleles were obtained by mixing wild-type DNA and mutant DNA.

Detection of KRAS mutations

Restriction fragment length polymorphism (RFLP) analysis

Mutations in the codon 12 of the *KRAS* (GenBank Accession Number NG_007524) were revealed as described earlier [19,20].

Single-strand conformation polymorphism

KRAS mutations were detected by nonisotopic SSCP using alkaline DNA denaturation and electrophoresis in nondenaturing polyacrylamide gel [21] with subsequent SYBR gold (dilution 1:10,000) staining.

Sequencing

The sequencing was performed by the fluorescent dideoxynucleotide termination method (Syntol, Moscow, Russia).

Real-time PCR and DMA protocols

The 55-bp *KRAS* fragment carrying codons 12 and 13 was amplified in 0.2-ml tubes or 96-well plates using an iQ5 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) in the presence of SYBR Green I (dilution 1:75,000). The primers KRAS-55-f (5′-ataaacttgtggccgttgg) and KRAS-55-r (5′-*biotin*-tgtatcgtcaagg ccctct) were designed to produce a 55-bp fragment of *KRAS*. The oligonucleotides KRAS-55-f and KRAS-55-r are modified at bases 13–14 (TA → CC) and 15 (A → C), respectively, to create two artificial *Hae*III restriction sites (see text). PCR amplification was carried out in a 25-μl sample containing 67 mM Tris–HCl buffer, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.25% (v/v) Tween 20, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 μM of each primer (Syntol, Moscow, Russia), 100 ng DNA, and 1 unit of *Taq* DNA polymerase (Syntol, Moscow, Russia). The PCR procedure consisted of an initial denaturation step at 95 °C for 5 min, followed by 10 cycles of 95 °C for 10 s, 50 °C for 20 s, and 30–32 cycles of 95 °C for 10 s, 58 °C for 20 s.

After the PCR, the DNA was heated at 95 °C for 3 min, cooled at a rate of 2 °C/s, and incubated at 40 °C for 3 min, after which it was melted from 50 to 90 °C (increment 0.5 °C, dwell time 25 s, rate of heating 3.3 °C/s) either immediately or after the sample optimization step (see below). The iQ5 Optical System software (version 2.1) was used to collect and analyze amplification and melting data from the iQ5 real-time PCR detection system. Fluorescence data (the negative derivative of fluorescence vs temperature) were exported to Microsoft Excel for normalization to the highest peak height.

Optimization of DMA conditions

This post-PCR procedure consisted of two consecutive stages: (i) amplicon digestion with the *Hae*III restriction enzyme (the 25-μl PCR sample was supplemented with 2.7 μl of 10× restriction buffer containing 500 mM NaCl, 100 mM $MgCl_2$, 100 mM Tris–HCl, pH 7.6, and 1 unit of *Hae*III restriction enzyme, followed by a 60-min incubation at 37 °C), and (ii) sample dilution with 3 vol of a standard solution containing 60 mM Na–phosphate buffer (pH 7 or pH 8), 3.5 mM Na–EDTA, and SYBR Green I (dilution 1:20,000). The resulting sample (final volume 110 μl) was melted under the conditions described above.

The KRAS-55-r primer is biotinylated at its 5′-end that makes it possible to use Streptavidin-coupled magnetic Dynabeads (Invitrogen Dynal AS, Oslo, Norway) for amplicon purification, if needed. In this case, the post-PCR 25-μl samples were supplemented with 3.5 μl 10× restriction buffer, 5 μl M-280 Dynabeads, and incubated for 40 min at room temperature with occasional mixing. Immobilized amplicons were transferred into fresh restriction buffer and digested with the restriction enzyme, and magnetic beads with remaining immobilized primers and 13-bp fragments were discarded. The subsequent procedures (sample dilution and melting) were carried out as described.

High-resolution melting

Primers KRAS-55-f and KRAS-55-r were used to amplify the 55-bp amplicon in 96-well plates using the CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). PCR was carried out in a 25-μl reaction volume containing SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA), 0.1 μM of each primer (Syntol, Moscow, Russia), and 100 ng human genomic DNA. The PCR was run under the following conditions: an initial denaturation step of 95 °C for 5 min, followed by 10 cycles of 95 °C for 10 s and 50 °C for 20 s, followed by 30 cycles of 95 °C for 10 s, and 58 °C for 20 s. After completion of amplification, DNA was heated at 95 °C for 3 min, kept at 40 °C for 3 min, and then melted from 50 to 90 °C (increment 0.3 °C, dwell time 12 s). The results were analyzed using the Bio-Rad Precision Melt Analysis software.

For optimization of the HRM analysis, the post-PCR 25-μl samples were supplemented with 1 unit *Hae*III restriction enzyme, incubated at 37 °C for 40 min, and diluted with an equal volume of 4× EvaGreen (Syntol, Moscow, Russia).

Results

In preliminary experiments, we tried to use snapback primers [12,13] for *KRAS* mutation scanning using the iQ5–SYBR Green I system. However, this approach, although very effective under the conditions of HRM, turned out to be unsatisfactory in the "low level" system (i.e., without appropriate equipment and software) because of a low signal-to-noise ratio. In the asymmetric PCR, the obligatory step in this method, numerous single-stranded molecules and nonspecific products are produced, causing a high