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Optimization of melting analysis with TaqMan probes for detection of *KRAS*, *NRAS*, and *BRAF* mutations

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ABSTRACT

The TaqMan probes that have been long and effectively used in real-time polymerase chain reaction (PCR) may also be used in DNA melting analysis. We studied some factors affecting efficiency of the approach such as (i) number of asymmetric PCR cycles preceding DNA melting analysis, (ii) choice of fluorophores for the multiplex DNA melting analysis, and (iii) choice of sense or antisense TaqMan probes for optimal resolution of wild-type and mutant alleles. We also determined ΔT_m (i.e., the temperature shift of a heteroduplex relative to the corresponding homoduplex) as a means of preliminary identification of mutation type. In experiments with serial dilution of mutant *KRAS* DNA with wild-type DNA, the limit of detection of mutant alleles was 1.5–3.0%. Using DNA from both tumor and formalin-fixed paraffin-embedded tissues, we demonstrated a high efficiency of TaqMan probes in mono- and multiplex mutation scanning of *KRAS, NRAS* (codons 12, 13, and 61), and *BRAF* (codon 600) genes. This cost-effective method, which can be applied to practically any mutation hot spot in the human genome, combines simplicity, ease of execution, and high sensitivity—all of the qualities required for clinical genotyping.

Individualized treatment of cancer is preceded by assessment of the genes that determine its clinical manifestations. Thus, the status of a number of clinically relevant genes (wild type or mutant) may in some cases determine the prognosis of the disease as well as sensitivity of the tumor to different therapeutic drugs [1–4]. In some common cancers, mandatory mutation scanning targets are *KRAS* and *NRAS* (codons 12, 13, and 61) as well as *BRAF* (mainly codon 600) genes defining the prognosis and treatment strategy for patients with colorectal cancer, lung cancer, and melanoma [4–14].

Sanger DNA sequencing, which until recently was the “gold standard” of clinical DNA diagnostics, began to give way to other methods [7,15,16] because of its low sensitivity (mutant allele detection limit of 15–20%) and, as a consequence, a large number of false-negative results. Simple, cost-effective, and sensitive methods are highly needed for mutation scanning in clinical laboratories. One such method that has become widely used recently is DNA melting analysis (DMA), which is often carried out in the high-resolution format (high-resolution melting analysis, HRMA) [7,17,18]. The theoretical basis of the method is the dependence of melting temperature (T_m) of the double DNA helix on its length, base composition (GC content), and sequence and the degree of complementarity of the strands [19]. Owing to the advent of “saturating” fluorescent DNA dyes and improved instrumentation, it became possible to identify single nucleotide polymorphisms (SNPs) in long (up to 1 kb) DNA sequences [18,20–23].

The DMA is widely used for mutation scanning of genes in which clinically significant mutations are found particularly often. The amplicons obtained in polymerase chain reaction (PCR) are usually assayed using unlabeled probes, snapback primers, or saturating DNA dyes [17,18,21,24–28]. Specific changes in the melting curves are indicative of mismatched bases in the duplexes and, hence, of mutations. This method is sensitive enough to detect a small proportion (~5%) of mutant DNA against a background of wild-type sequences [5,17]. An important advantage of HRMA is the “closed tube” format, which is simple and rapid and excludes sample cross-contaminations.

It was demonstrated recently that self-quenched dual-labeled (TaqMan) probes can be used not only for monitoring the PCR in

Abbreviations: DMA, DNA melting analysis; HRMA, high-resolution melting analysis; T_m, melting temperature; PCR, polymerase chain reaction; FFPE, formalin-fixed paraffin-embedded.

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real time, which was what they were intended for in the first place [29], but also for the DMA [30]. Fluorescence of the probe was shown to increase not only when it is cleaved by *Taq* polymerase in PCR but also when it is hybridized with the template (in both cases, the fluorophore and the quencher became spatially separated) (Fig. 1). This circumstance allows using TaqMan probes in the "closed" format—first performing asymmetric PCR (to accumulate single-stranded DNA) and then the DMA immediately after it.

Although TaqMan probes are not as versatile as the intercalating dyes (e.g., SYBR Green I, EvaGreen), they have a host of other advantages such as the possibility of performing multiplex analysis, high specificity, and high sensitivity. The latter is due to the fact that the very short (25- to 40-bp) duplexes between TaqMan probes and single-stranded DNA produce a strong mismatch-induced temperature shift on melting. Besides, the melting curves of TaqMan probes better lend themselves to interpretation because they arise from one homoduplex and one heteroduplex, whereas intercalating dyes produce a superposition of melting of two homoduplexes and two heteroduplexes. Thus, the TaqMan probes, already widely used in real-time PCR, have the advantages of versatility, multiplexing, and high flexibility in probe design. Because the closed format minimizes the risk of sample cross-contaminations, the TaqMan probes seem to be ideally suited for a simple, cost-effective, and sensitive DNA melting analysis of clinical specimens.

In this work, we studied various PCR parameters that affect efficiency of this method. After optimization of the experimental conditions, the DMA was employed in mono- and multiplex scanning of clinically significant *KRAS, NRAS,* and *BRAF* genes.

Materials and methods

Samples

Tumor tissue samples (lung cancer, colon cancer, and melanoma) were obtained from the N. N. Blokhin Russian Cancer Research Center. The DNA from tumor samples was isolated by phenol–chloroform deproteinization and from formalin-fixed paraffin-embedded (FFPE) tissues by using the QIAamp DNA FFPE Tissue Kit (Qiagen) as recommended by the manufacturer. DNA concentration was determined spectrophotometrically (Nano-Drop 1000, Thermo Scientific).

The human colon carcinoma cell line SW480 with mutated codon 12 (GGT → GTT) [31] was kindly provided by M. Yakubovskaya (Institute of Carcinogenesis, Moscow, Russia). Different ratios of the alleles were obtained by mixing wild-type DNA and mutant DNA.

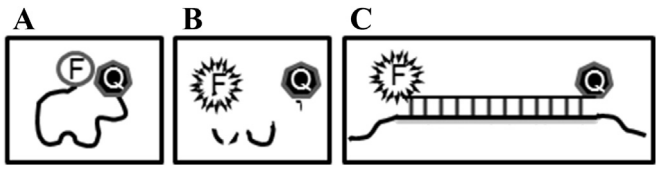

Fig.1. Schematic presentation of various states of the TaqMan probe [30]. A A random coil in aqueous solution is weakly fluorescent because fluorescence quenching arises due to the proximity of the fluorophore (F) and quencher (Q). B The TaqMan probe annealed to the template is hydrolyzed by the 5′-to-3′ exonuclease activity of the *Taq* polymerase during asymmetric PCR, and the fluorophore released from association with quencher becomes strongly fluorescent. C On completion of asymmetric PCR, the TaqMan probe hybridizes with excessive target strands and becomes strongly fluorescent because the fluorophore and quencher are spatially separated (after thermal denaturation, the probe returns back to weakly fluorescent state). The duplex of the TaqMan probe with fully matched wild-type target is more stable than that with a mismatch what is reflected in the corresponding melting curves.

Sanger sequencing

Bidirectional analysis was performed by the fluorescent dideoxynucleotide termination method (Syntol, Moscow, Russia).

Asymmetric real-time PCR and DNA melting

Primers and probes used for *KRAS, NRAS,* and *BRAF* analysis are presented in Table 1. The alignments of the primers, probes, and mutation "hot spots" along the amplicons studied are presented schematically in Fig. 2.

Amplification reactions were carried out in 96-well plates in a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). Each 25-μl reaction contained 50 mM Tris–HCl (pH 8.8), 50 mM KCl, 0.01% (v/v) Tween 20, 3 mM $MgCl_2$, 0.25 mM deoxynucleoside triphosphates (dNTPs), 1.25 U of *Taq* polymerase, a primer pair (0.04/0.4 μM), 0.2 μM TaqMan probe (Syntol), and 5 μl of DNA template. Real-time PCR protocols started with a denaturation step for 5 min at 95 °C, followed by 55 cycles of 95 °C for 13 s, 62 or 57 °C (for *KRAS* or *NRAS–BRAF*, respectively) for 15 s, and 72 °C for 20 s, with fluorescence acquisition at 72 °C.

After the PCR, the DNA was heated at 95 °C for 1 min, cooled at a rate of 2 °C/s, and incubated at 50 °C for 2 min, after which it was melted from 50 to 85 °C (increments of 0.4 °C, dwell time of 6 s, rate of heating of 3.3 °C/s). The Bio-Rad CFX96 Manager software (version 1.6) was used to collect and analyze amplification and melting data from the CFX96 real-time PCR detection system. In some cases, fluorescence data (the negative derivative of fluorescence vs. temperature) were exported to Microsoft Excel for normalization to the highest peak height.

Results and discussion

Unlike some other methods of mutation scanning that require complex experimental setting [32,33], the method used in the current study is quite economical because the widely used TaqMan probes are the routine components of real-time PCR [30]. Nonetheless, some DMA conditions need optimization, and we selected *KRAS* for this purpose as one of the most clinically significant genes.

Number of asymmetric PCR cycles

A prerequisite of this approach is asymmetric PCR producing single strands, which are the targets of TaqMan probes [30]. In preliminary experiments, we noted that an increase in the number of cycles of asymmetric PCR (40, 55, or 70 cycles) often does not result in the expected increase in the melt peaks, and sometimes they are even surprisingly reduced. It was suggested that after reaching the amplification plateau, complementary DNA strands rapidly anneal to one another and fall out of the reaction, whereas the single-stranded DNA, being in excess, continues to be available to the appropriate primer and is converted into the double-stranded form. As a result, the concentration of the single-stranded DNA in asymmetric PCR after reaching a plateau may decrease. In addition, an excess (above a certain optimum) of asymmetric PCR cycles leads to distortion of the melt peaks (probably due to the accumulation in the reaction medium of unfinished strands resulting from premature detachment of the enzyme approaching the 5′ end of the template [34]), which can be mistaken for a mutation (Fig. 3). Although the number of cycles of asymmetric PCR must, in general, be optimized individually because reaching the reaction plateau will vary depending on a variety of conditions, in our hands in most cases 55 cycles gave acceptable results.