Short Communication

Evaluation of High-Resolution Melting (HRM) for Mutation Scanning of Selected Exons of the *CFTR* Gene

(*CFTR* / high-resolution melting / HRM / mutation detection)

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Abstract. Hereby we present evaluation of high-resolution melting for mutation scanning applied to the cystic fibrosis transmembrane conductance regulator gene. High resolution melting was used for mutation scanning of selected samples derived from cystic fibrosis patients with a known cystic fibrosis transmembrane conductance regulator genotype. We tested 19 different disease-causing cystic fibrosis transmembrane conductance regulator mutant genotypes located within six exons of the cystic fibrosis transmembrane conductance regulator gene (4, 7, 10, 11, 14b and 22). Normalized melting curves of tested samples were compared to sequenced-verified wild-type samples. Determined mutations are as follows: p.F508del, p.I507del, p.G551D, p.R347P, c.1717-1G>A, c.621+1G>T, p.Y122X, p.I336K, p.R553X, c.2789+5G>A, c.574delA, c.1811+1G>C, p.L1335F, p.L1335P, p.L1324P and p.M470V and represent minimally 76.5 % of all cystic fibrosis alleles detected in the Czech cystic fibrosis population. All analysed samples with mutant genotypes were unambiguously distinguished from wild-type samples. High-resolution melting analysis enabled reliable detection of all single-nucleotide polymorphism classes and 1- or 3-base pair deletions. We examined the specificity, sensitivity and precision of this methodology. High-resolution melting analysis is an economical, sensitive and specific close-tube method and has a high utility for the detection of unknown mutations in cystic fibrosis DNA diagnostics.

Introduction

To date, more than 1,600 mutations have been identified in the cystic fibrosis transmembrane conductance regulator (*CFTR)* gene (http://www.genet.sickkids.on.ca/cftr). The *CFTR* gene consists of 27 exons (Riordan et al., 1989), thus making the detection of non-common mutations by sequencing laborious, expensive and time-consuming. To simplify the analysis of such a broad mutation spectrum, a rapid and reliable method is required. There are many available scanning methods, such as single-strand conformation polymorphism analysis (SSCP) (Orita et al., 1989), denaturing gradient gel electrophoresis (DGGE) (Lerman and Silverstein, 1987), temperature gradient capillary electrophoresis (TGCE) (Li et al., 2002), denaturing high-performance liquid chromatography (dHPLC) (Xiao and Oefner, 2001) or heteroduplex analysis (HA) (Highsmith et al., 1999) which are time-consuming and the sensitivity often depends on the experience of the operator. On the other hand, HRM presents a rapid, high-throughput, closed-tube method for mutation scanning and genotyping (Wittwer et al., 2003). The sample preparation consists of a standard PCR reaction with a dsDNA intercalation fluorescent dye and does not require any post-PCR handling. Products can be analysed directly after PCR amplification using specially designed instruments for high-resolution melting (HRM) analysis. The homozygous, heterozygous and wild-type samples are differentiated according to their melting profile, which is represented by plotting fluorescence over the temperature range. The heterozygous genotype is distinguished from a wild-type sample by different melting temperatures (T_m) and the shape of the melting curve, whereas homozygous genotypes are distinguished only by a change in T_m.

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Abbreviations: CF – cystic fibrosis, CFTR – cystic fibrosis transmembrane conductance regulator, DGGE – denaturing gradient gel electrophoresis, dHPLC – denaturing high-performance liquid chromatography, HA – heteroduplex analysis, HRM – high-resolution melting, PCR – polymerase chain reaction, SSCP – single-strand conformation polymorphism analysis, SNP – single nucleotide polymorphism, TGCE – temperature gradient capillary electrophoresis, T_m – melting temperature.

Many publications have documented the successful use of HRM for mutation scanning and genotyping (Wittwer et al., 2003; Liew et al., 2004; Reed and Wittwer, 2004; Zhou et al., 2004, 2005; Chou et al., 2005; Graham et al., 2005; Krypuy et al., 2006, 2007; Montgomery et al., 2007; Audrezet et al., 2008, Nguyem-Dumont et al., 2009). HRM is a mutation detection and scanning technique that has high reliability. It has been reported to have near 100% sensitivity and specificity when the analysed PCR products were up to 400 bp in length (White and Potts, 2006).

To evaluate the reliability of HRM in our laboratory, we tested the specificity, sensitivity and repeatability in detecting 16 representative mutations (19 genotypes) within six exons of the *CFTR* gene. In comparison to previously published studies we focused on the utilization of Rotor-Gene™ 6000. This instrument has a specially tuned high-intensity optical channel and extreme thermal resolution (± 0.02 °C). Due to the unique rotary design there is the highest thermal uniformity between samples (± 0.01 °C), hence there is no need for temperature shifting. Compared to most block-based systems the light in our case is highly focused, while in the block-based systems it is most intense in its centre and becomes dispersed on the edges. This leads to optical variability and non-uniformity within the analysed plate. We also used the IdahoTechnology dye, which provides highest sensitivity compared to all subsequent proprietary dyes from other companies. All these properties make this technical platform unique and highly suitable for the diagnostic setting.

Material and Methods

DNA samples

We selected clinical samples derived from CF patients of our Institute with known genotypes previously determined by sequencing analysis. Genomic DNA was extracted from leukocytes of peripheral blood using a commercial PUREGENE DNA Purification Kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions or by the "salting out" procedure. All DNA samples were diluted in the same buffer PUREGENE DNA Hydration Solution (Gentra Systems) at the concentration 15 ng/µl.

Polymerase chain reaction conditions

Human genomic DNA was amplified by polymerase chain reaction (PCR) using previously published primers (Macek et al., 1997) in the presence of the intercalating fluorescent dye LCGreen plus (Idaho Technology, Salt Lake City, UT). Amplification efficiency was monitored using real-time PCR. PCR reactions were performed in 10 µl reaction volume which consisted of 1× PCR buffer including 2 mM $MgCl_2$ (final concentration), 0.3 µM of each primer, 1 mM of dNTPs, 1× LCGreen plus, 1 U of FastStart Taq DNA Polymerase (Roche Diagnostics, Indianapolis, IN) and 30 ng of genomic DNA. To determine the precision, all PCR reactions were performed five times.

PCR cycling and HRM analysis were performed in Rotor-Gene™ 6000 (Corbett Life Science, Qiagen, San Francisco, CA). The amplicons were run according to the following conditions: one cycle of initial denaturation at 95 °C for 4 min, followed by 40 cycles of 95 °C for 20 s, annealing for 30 s, 72 °C for 40 s and one cycle of final extension at 72 °C for 2 min. The annealing temperature was 62 °C for amplicons 10, 11, 14b and 22, 67 °C for amplicon 4 and 58 °C for amplicon 7. Following amplification, PCR products were denatured at 95 °C for 1 min and rapidly cooled to 25 °C for 1 min to form heteroduplexes.

Melting conditions

Melting analysis was performed immediately after PCR in the same instrument. The fluorescence signal was acquired from 65 °C to 95 °C at a ramp rate 0.1 °C/s. Melting data were visualized and analysed using Rotor-Gene™ 6000 Series Software 1.7 (Corbett Life Science). Melting curves of examined samples were normalized and the difference temperature graphs were compared against wild-type control samples.

Results and Discussion

We scanned 19 different mutant genotypes (Table 1) located within six exons of the *CFTR* gene (4, 7, 10, 11, 14b and 22). Analysed mutations included all SNP classes (which divided SNPs into four groups according to the intensity of T_m change) and 1- or 3-base pair deletions and represent at least 76.5 % of all CF alleles detected in the Czech Republic.

Evaluated amplicons varied in size from 101 bp to 380 bp and had a GC content ranging from 33.7 % to 45.9 %. The tested samples were evaluated by initial visual inspection of melting curves and by software analysis. All examined samples with mutant heterozygous genotype were unambiguously distinguished from wild-type samples by a different shape of the melting curves (Figs 1A, 1B, 1C, 1D, 1E, 1F, 1G and 1H). Heterozygous profiles of p.G551D versus p.R553X and p.L1335F versus p.L1335P were not distinguishable (Figs. 1A and 1B).

A sample with homozygous genotype both for p.F508del and p.M470V mutations showed a similar melting pattern as the wild-type control samples and the T_m shift not easily distinguished (Fig. 1C). The presence of the homozygous mutations was confirmed by mixing (1 : 1) the PCR product with the wild-type PCR product, denaturing and reanalysing the melting. In that way, heterozygosity of both mutations was established and the detection of the homozygous mutations by melting analysis was obvious – the melting pattern was similar as the original sample with heterozygous genotype p.M470V/p.F508del (Fig. 1D).

We scanned nine samples for exon 4 (three heterozygous, six wild-type controls), eight samples for exon 7 (four heterozygous, four wild-type controls), nine samples for exon 10 (four heterozygous, one homozygous

Table 1. CFTR mutations analysed in the study

Exon	Genotype	Nucleotide Change	SNP class*
4	c.621+1G>T	G>T	2
	p.Y122X	T>A	4
	c.574delA	del	--
7	p.R347P	G>C	3
	p.I336K	T>A	4
10	p.F508del/p.M470V/p.M470V	del/A>G/A>G	1
	p.F508del/p.F508del/p.M470V/p.M470V	del/del/A>G/A>G	1
	p.F508del/p.M470V	del/A>G	1
	p.M470V	A>G	1
	p.I507del	del	--
11	c.1717-1G>A	G>A	1
	c.1811+1G>C	G>C	3
	c.1717-1G>A / c.1811+1G>C	G>A / G>C	1/3
	p.G551D	G>A	1
	p.R553X	C>T	1
14b	c.2789+5G>A	G>A	1
22	p.L1335F	C>T	1
	p.L1335P	T>C	1
	p.L1324P	T>C	1

* (Venter et al., 2001)
SNP class 1 represents C/T and G/A base changes with typical T_m melting curve shift larger than 0.5 °C. SNP class 2 represents C/A and G/T base changes, class 3 C/G base change with T_m melting curve shift between 0.5–0.2 °C. SNP class 4 comprises A/T base change, which caused T_m melting curve shift smaller than 0.2 °C.

and four wild-type controls), twelve samples for exon 11 (seven heterozygous, five wild-type controls), seven samples for exon 14b (two heterozygous, five wild-type controls) and seven samples for exon 22 (three heterozygous, four wild-type controls). To determine the precision, each sample was prepared five times. In total, we analysed 120 wild-type melting curves and 140 mutant melting curves with 100% sensitivity and 96% specificity.

Currently, there is discussion whether mutation scanning techniques still have a role in DNA diagnostics, since many argue that lowering costs for sequencing render these unnecessary. However, even in the case of sequencing, false positivity/negativity could occur if one does not sequence both strands. Usually, in a routine diagnostic setting, laboratories only sequence one strand – an approach associated with higher risk of error. We have confirmed this contentious issue (currently in press) within our participation in the Eurogentest (www.eurogentest.org) consortium. Moreover, next-generation sequencing techniques still do not have the desirable sensitivity and specificity, since enrichment strategies are not yet optimized for DNA diagnostics (Hert et al., 2008; Voelkerding et al., 2009).

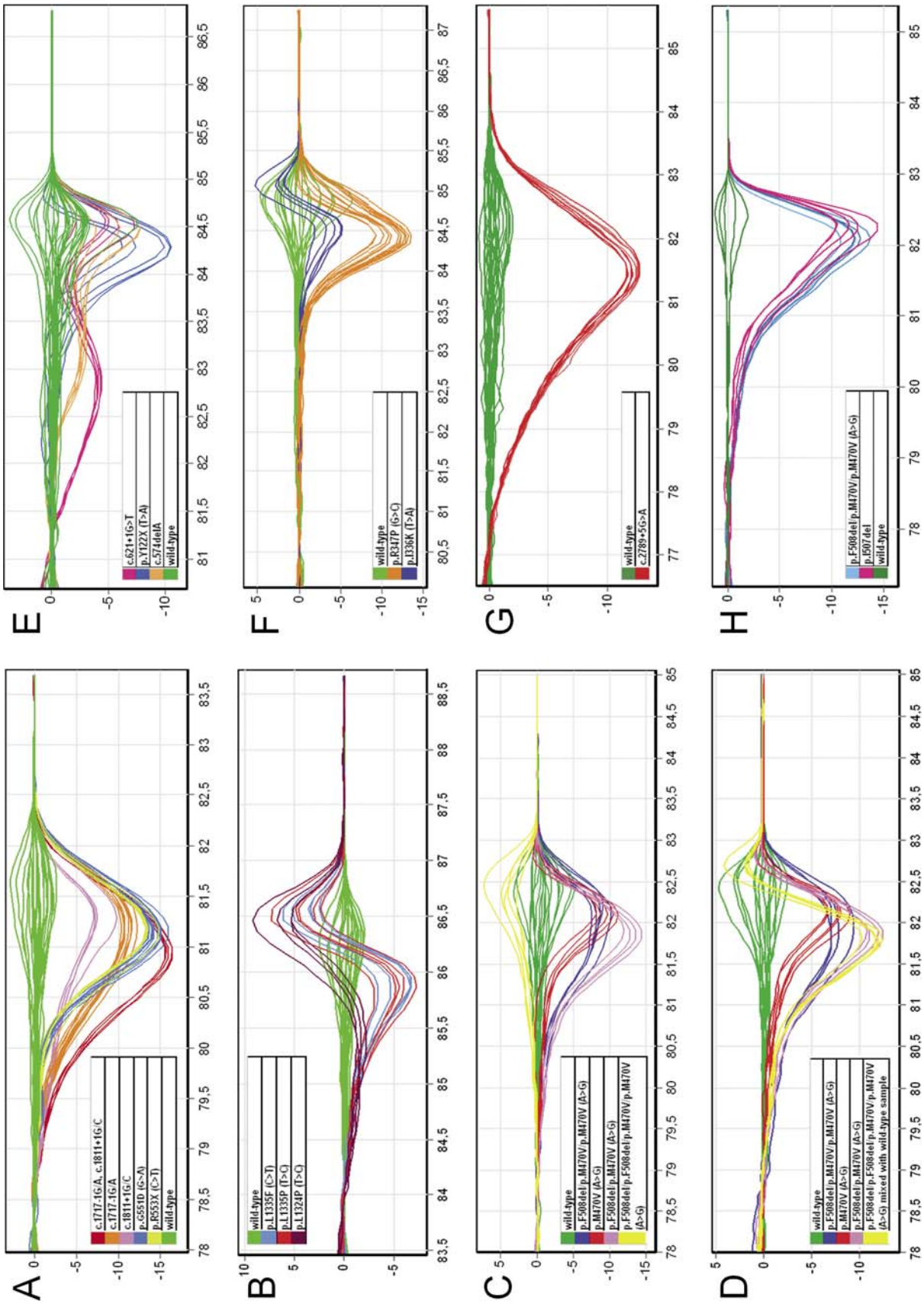
Therefore, there is still space for mutation pre-scanning prior to sequencing the "positives", as we have proved in the case of HRM. In this respect, we have studied a particular technical variant of HRM, based on the RotorGene™ 6000 device and the original HRM dye developed by IdahoTechnology. In this way we accounted for previously described disadvantages of alternative HRM platforms.

In our hands HRM allowed easy detection of all SNP classes as well as 1- and 3-base pair deletions. Heterozygous mutations belonging to the first SNP class cause the biggest change of T_m and should thus be the easiest mutations to detect. We detected this group very clearly, and we were also able to simply identify the most difficult fourth SNP class (Figs. 1E and 1F). The detection of homozygous mutations is complicated since generally the T_m difference is not high enough to allow proper discrimination. By generating "artificial" heterozygous samples from the 1 : 1 mixture, we were able to accurately detect homozygous sample.

All analysed samples with heterozygous mutant genotypes were unambiguously distinguished from wild-type samples. This method exhibits very high specificity and sensitivity, making it suitable for its use as a pre-screening method in diagnostics.

In some cases false positives appeared, hence we reached an almost 96 % of specificity. This could have been caused by analysing various old DNA samples, which were isolated by different techniques. Various DNA storage solution buffers can affect the melting behaviour in HRM, leading to broader dispersal of melting profiles, and thus could have contributed to false-positive "calls" by the software. It is also possible that the observed false-positive rates could be lowered following further optimization. Nevertheless, in diagnostic setting there is usually no time to perform such optimization, since it is expected that the technique should provide uniform results.

Figs. 1A–1H demonstrate the repeatability of the method; the melting curves of each sample were prepared in one day, by one analyst in the same instrument. Excellent repeatability is demonstrated by the overlapping curves of multiple samples for both the wild-type and the mutant samples.



Figs 1A-1H. Normalized difference graphs. Each genotype was melted and displayed in five replicates. A – exon 11. B – exon 22. C – exon 10. D – exon 10; homozygous sample (yellow colour) was mixed 1 : 1 with wild-type PCR product, denatured and reanalysed. E – exon 4. F – exon 7. G – exon 14b. H – exon 10.

In conclusion, HRM analysis is an economical, sensitive and specific close-tube method that can dramatically reduce the need for sequencing. Consequently, it has a high utility for the detection of unknown mutations in CF DNA diagnostics. The only caveat, which pertains to all other PCR-based techniques, is the quality of template DNA to which one needs to pay extra attention. We hope that our experience could be applicable to other HRM diagnostic applications.

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