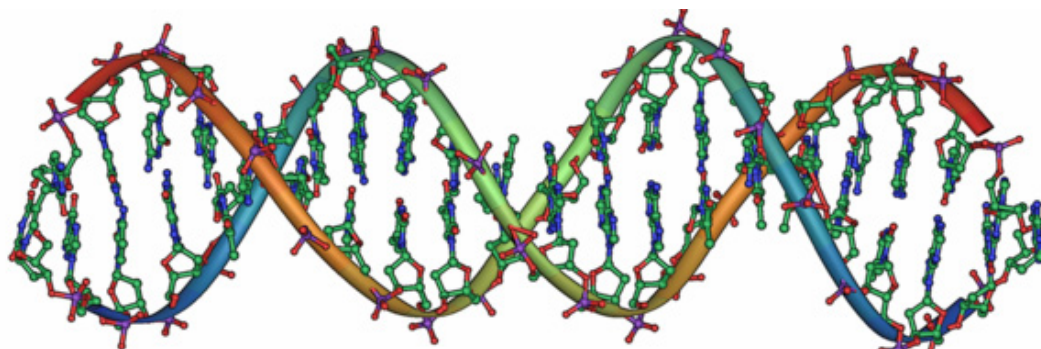


Discrimination of base-pair neutral single nucleotide polymorphism (SNP) by high-resolution melting on QuanTyper™-48



High-resolution melting (HRM) analysis has recently emerged as a simple, rapid and cost-effective method for mutation screening and genotyping of single nucleotide polymorphisms. In this study, we show that the most subtle and hard to detect genetic variation, an A/T conversion, is possible to genotype by HRM on QuanTyper-48.

Introduction

High-resolution melting (HRM) analysis is a novel method for studying genetic variation within PCR amplicons. The technique was initially thought to require the use of a new generation of saturating DNA dyes¹, but was recently shown to work equally well with SYBR Green I, a non-saturating dye^{2,4}. In HRM-designated instruments the decrease in fluorescence caused by the transition of dsDNA to single stranded DNA (ssDNA) with increasing temperature is carefully monitored. With the aid of tailor-made analysis software, different genetic variants can be discerned by the appearance of their characteristic melting curves. The fact that probes are not required makes experiments less costly as well as easier to set up and optimize compared to conventional PCR-based genotyping techniques.

The usefulness of HRM for genotyping of single nucleotide polymorphisms

(SNPs) has been demonstrated in several recent reports⁵⁻⁷. Amplicons containing the polymorphic loci are generated and the different genetic variants can be identified by the appearance of their respective melting profiles. Homozygotes are distinguished from each other by a shift of the melting curve along the temperature axis while heterozygotes differ from homozygotes in curve shape. One of the most challenging tasks for an HRM-enabled instrument is the discrimination of base-pair neutral homozygotes, i.e. A/T to T/A, or, C/G to G/C conversions⁸. The difference in melting temperature between AA and TT homozygotes is approximately 0.1 °C and highly sensitive instrumentation is required in order to separate their melting curves. In this study, the aim was to show that HRM on QuanTyper-48 super-convective^{9,10} thermal cycler is sufficiently sensitive and accurate to discriminate between two such amplicons.

Materials and Methods

Two 50 base pair oligonucleotide pairs corresponding to the Y-chromosomal region NCBI36:Y:20327149-20327198, containing either A:T or T:A in position 23 functioned as templates for the PCR reaction. Primers M45F and M45R, previously designed to amplify this genomic region and subsequently confirmed to also amplify the artificial template were used in the PCR reaction. Real-time PCR was performed in QuanTyper-48 according to the conditions below. Immediately following thermal cycling, high-resolution melting profiles were generated by subjecting the samples to a temperature gradient while continuously monitoring the drop in fluorescence as the nucleotide strands denatured.

The high-resolution melting data were analyzed using QT analysis software v. 1.03. The raw fluorescence data was subjected to normalization and temperature shifting in order to remove background fluorescence, to make up for sample-to-sample variation and to aid visual interpretation and automatic grouping of similar melting curves. Normalization intervals of approximately 2 °C were set in linear regions before and after the melting transition and the curves were rescaled from 0 to 100 % fluorescence. Automatic grouping of similar melting curves was carried out using a shape-matching algorithm within the analysis software.

The samples were run in duplicates on three separate occasions with consistent results.

Table 1. PCR protocol

	Final conc.
Oligo template	1 nM
primer M45F	0.4 µM
primer M45R	0.4 µM

MgCl ₂ [†]	2.5 mM
10x Buffer [†]	1x
Taq polymeras [†]	0.04 U/µL
SYTO 9 [†]	2 mM
dNTPs [‡]	0.2 Mm
dH ₂ O to a final volume of	20 µl

PCR cycling parameters

95 °C for 120 s followed by 35 cycles of [95 °C for 0 s; 60 °C for 3 s; 72 °C for 8 s].

HRM parameters

65 °C for 10 s, followed by a temperature gradient from 65 ° to 85 ° at a rate of 0.1 °C/s.

Results and Discussion

After normalization and temperature shifting of the raw fluorescence data a slight shift along the temperature axis made the two genetic variants easily distinguishable by visual inspection (Figures 1 and 2). Minimal spread between duplicates was observed in all runs (Figure 1). A combination of the two genotypes A and T, representing a heterozygous sample, was also clearly distinguishable as a third curve shape (dark green, Figure 2).

SuperConvection™ is a novel technology that minimizes thermal heterogeneity within samples by inducing enhanced mass-transport in the reaction mixture. Together with a sophisticated in-tube temperature measurement system, this enables an exact control of sample temperature which can potentially increase the sensitivity and accuracy of HRM. Moreover, the positive effect of SuperConvection on temperature homogeneity enables a continuous heat gradient, resulting in a higher melting curve resolution compared to other HRM instruments that rely on stepwise heating.

[†] From Invitrogen.

[‡] Each dNTP at 0.2 mM final conc. dNTPs from GE Healthcare.

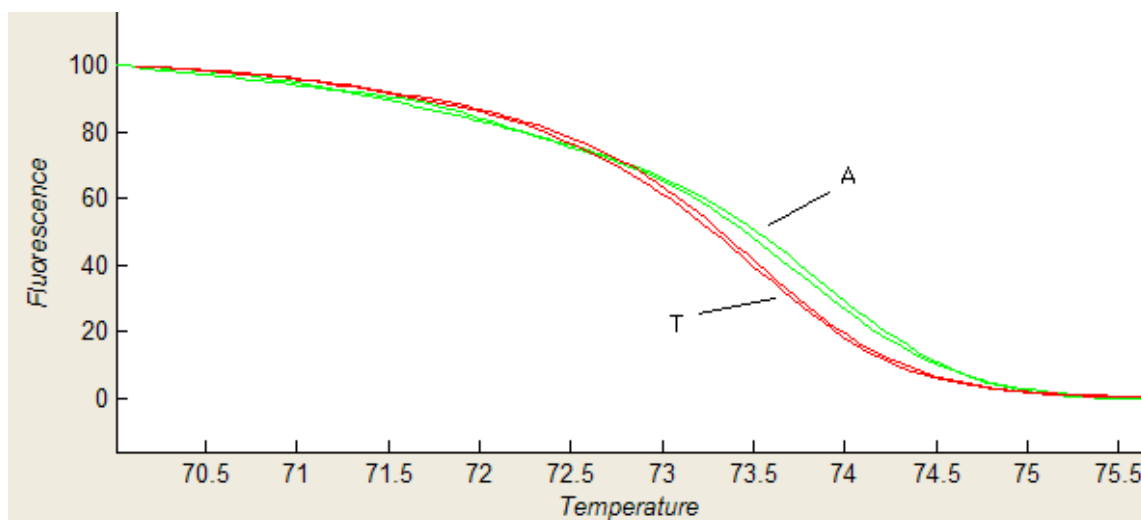


Figure 1 HRM analysis on QuanTyper-48 discriminating A from T at a Y-chromosomal SNP position.

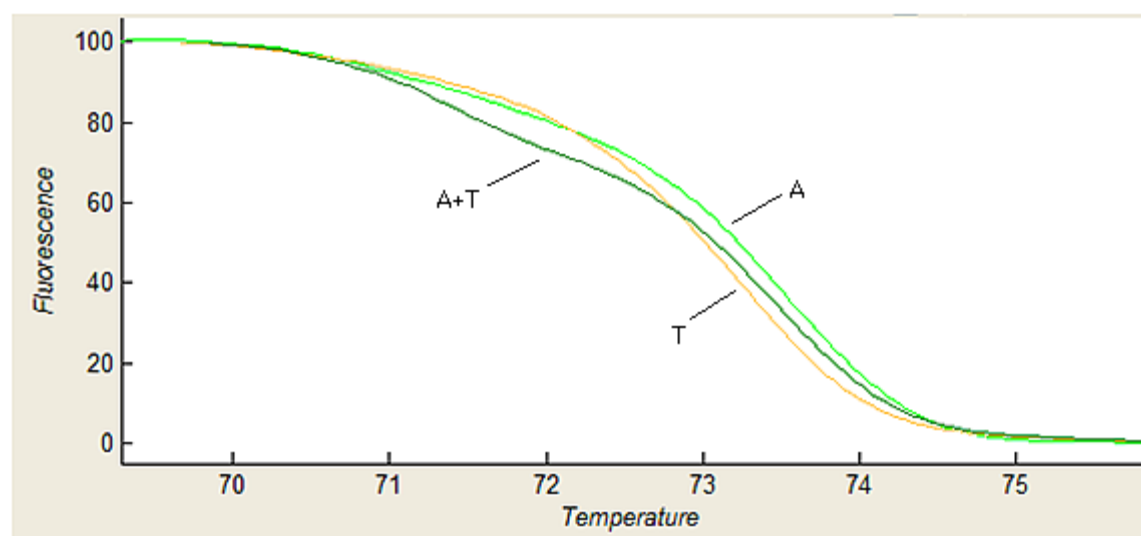


Figure 2 HRM analysis on QuanTyper-48 using the same Y-chromosomal SNP PCR as in Figure 1. A is again discriminated from T. In addition, an A+T mix (equivalent to a heterozygous sample of an autosomal locus) is clearly distinguishable as a third curve shape (dark green).

The performance of QuanTyper-48 was tested by subjecting it to the most demanding task for an HRM-enabled instrument, namely the discrimination of base-pair neutral homozygotes. By successfully distinguishing the melting

curves of two amplicons differing by a single A/T SNP, it is concluded that HRM on QuanTyper-48 has the capability to challenge the leading instruments on the market in terms of sensitivity and accuracy.

References

1. Wittwer CT, GH Reed, CN Gundry, JG Vandersteen, and RJ Pryor. 2003. High-resolution genotyping by amplicon melting analysis using LCGreen. *Clin. Chem.* 49:853-860.
2. Dufresne SD, DR Belloni, WA Wells, and GJ Tsongalis. 2006. BRCA1 and BRCA2 mutation screening using SmartCycler II high-resolution melt curve analysis. *Arch. Pathol. Lab. Med.* 130:185-187.
3. Price EP, H Smith, F Huygens, and P M Giffard. 2007. High-resolution DNA melt curve analysis of the clustered, regularly interspaced short-palindromic-repeat locus of *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 73:3431-3436.
4. Pornprasert S, A Phusua, S Suanta, R Saetung, and T Sanguansermisri. 2008. Detection of alpha-thalassemia-1 Southeast Asian type using real-time gap-PCR with SYBR Green1 and high-resolution melting analysis. *Eur. J. Haematol.* 80:510-514.
5. Palais RA, MA Liew, CT Wittwer. 2005. Quantitative heteroduplex analysis for single nucleotide polymorphism genotyping. *Anal. Biochem.* 346(1):167-75.
6. Kristensen LS, and A Dobrovic. 2008. Direct genotyping of single nucleotide polymorphisms in methyl metabolism genes using probe-free high-resolution melting analysis. *Cancer Epidemiol. Biomarkers Prev.* 17(5):1240-7.
7. Hung CC, CN Lee, CH Chang, YJ Jong, CP Chen, WS Hsieh, YN Su, and WL Lin. 2008. Genotyping of the G1138A mutation of the FGFR3 gene in patients with achondroplasia using high-resolution melting analysis. *Clin. Biochem.* 41(3):162-6.
8. Liew M, R Pryor, R Palais, C Meadows, Erali M, Lyon E. and C Wittwer. 2004. Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. *Clin. Chem.* 50:1156-1164.
9. Malmqvist, M. 2004. Homogenizing of small-volume mixtures by centrifugation and heating. United States Patent 6,783,993.
10. Mårtensson, G., Skote, M., Malmqvist, M., Falk, M., Asp, A., Svanvik, N. and Johansson, A. 2006. Rapid PCR amplification of DNA utilizing Coriolis effects. *European Biophysics Journal* 35 (6): 453-458.

Primers were kindly provided by Dr. Marie Allen, Dept. of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, Sweden.

SYTO 9[®] and Platinum Taq[®] are registered trademarks of Invitrogen.



AlphaHelix Molecular Diagnostics AB [publ]
Kungsängsv. 29, SE-753 23, Uppsala, Sweden
Phone +46 18 120701. Fax +46 18 120 703
E-mail: info@alphahelix.com
Web: www.alphahelix.com