

Gene Scanning by High-Resolution Melt (HRM) on a LightCycler® 480 Real-Time PCR System Using EXPRESS SYBR® GreenER™ qPCR SuperMix

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Introduction

High-resolution melt (HRM) is a recent enhancement to traditional DNA melting analysis that can be used to differentiate sequence variations within PCR amplicons. HRM is used to characterize samples according to their dissociation profile as they transition from double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA). In addition, it is a simple, closed-tube system that offers a more cost-effective method for characterizing samples than fluorescent probe-based methods. Some widely used applications for HRM are mutational analysis (including genotyping)¹, gene scanning, and methylation analysis.²

HRM requires initial amplification of the target sequence by PCR in the presence of a DNA-binding dye that generates a strong fluorescent signal only when bound to dsDNA. The

PCR amplicons are then melted by slowly increasing the temperature. The amplification takes place in a real-time qPCR instrument, and fluorescence is monitored throughout. As dsDNA transitions to ssDNA, there is a sharp decrease in fluorescence. This dissociation profile is specific for a given DNA sequence. Mutations are detected as either a shift in the melting temperature (T_m) or a change in the shape of the melting curve.

In this application note, we present the results of a gene scanning experiment performed on a LightCycler® 480 Real-Time PCR System (Roche Applied Science) using EXPRESS SYBR® GreenER™ qPCR SuperMix to scan four human single nucleotide polymorphisms (SNPs).

Materials and methods

qPCR SuperMix

EXPRESS SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen Cat. no. 11784-200) was used for PCR and subsequent HRM analysis. The supermix contains all the components necessary for PCR and HRM except primers and template. The SYBR® GreenER™ dye provides greater sensitivity and reduced PCR inhibition compared to other fluorescent dsDNA-binding dyes and, as such, shows particular utility in HRM analysis.

Table 1—SNPs for gene scanning. One SNP from each of the four SNP classes was used for gene scanning.

Gene symbol	SNP class	Mutation	Frequency ³ (in the human genome)	Amplicon length (bp)
ABCC8	1	A/G	0.662	145
CYP19A1	2	C/G	0.176	123
UGT1A1	3	G/T	0.088	143
ABCC1	4	A/T	0.074	120

Primers

PCR primers for four SNPs (Table 1) were designed using Primer Express® Software Version 3.0 (Applied Biosystems). Primers were designed to have annealing temperatures in the range of 58° to 60°C and to produce amplicons in the range of 100 to 150 bp. A concentrated 20X (4 μM each) primer mix was made for each primer set. The final concentration of primers in PCR was 0.2 μM each.

Table 2—Reaction mix setup.

Component	Volume	Final concentration or amount
2X EXPRESS SYBR® GreenER™ SuperMix	5.0 μl	1X
20X primer mix	0.5 μl	0.2 μM (each primer)
Template DNA (2.5 ng/μl)	2 μl	5 ng
DEPC H ₂ O	2.5 μl	–
Total volume	10 μl	

Table 3—Cycling protocol.

Cycling protocol				
Detection format		Block type		Reaction volume
SYBR® Green I dye		384		10 μl
Programs				
Program name	Cycles		Analysis mode	
Preincubation	1		None	
Amplification	45		Quantitation	
High-resolution melt	1		Melting curve	
Cooling	1		None	
Temperature targets				
Target (°C)	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/sec)	Acquisitions (per °C)
Preincubation				
95	None	00:00:20	4.8	–
Amplification				
95	None	00:00:01	4.8	–
60	Single	00:00:20	2.5	–
High-resolution melt				
95	None	00:01:00	4.8	
40	None	00:01:00	2.5	–
55	None	00:00:01	1	
95	Continuous	–	–	25
Cooling				
40	None	00:00:10	2.5	–

Template DNA

Human genomic DNA samples were purchased from the Coriell Institute for Medical Research. Twenty DNA samples of unknown genotypes for the above SNPs were chosen for gene scanning from the Human Variation Panel - Caucasian Panel of 50 (Coriell Cat. no. HD50CAU). Five nanograms of each DNA sample were used as template in the PCR.

PCR protocol

The protocol presented here has been optimized for fast-cycling qPCR using the reagents in the EXPRESS SYBR® GreenER™ qPCR SuperMix Universal on a LightCycler® 480 Real-Time PCR System with a 384-well plate. See Tables 2 and 3 for details on the reaction mix and cycling parameters.

Data analysis

Data analysis was performed using the LightCycler® 480 Gene Scanning Software. Melt curves were fluorescence-normalized and temperature-shifted (Figure 1). The software was set to autogroup samples into Common and Variant genotypes (data not shown).

Results and conclusions

The most widely used high-resolution melt (HRM) application is gene scanning. Gene scanning is the search for the presence of unknown variations in PCR amplicons prior to, or as an alternative to, sequencing. Mutations in PCR products are detectable by HRM analysis by a change in the shape of the DNA melting curves. When amplified and melted, heteroduplex DNA samples exhibit melting curves with different profiles than those derived from homozygous wild-type or mutant samples.

The results presented here show that EXPRESS SYBR® GreenER™ qPCR SuperMix was used successfully in a gene scanning experiment (Figure 1). All four assays were able to clearly and correctly differentiate heterozygous samples from homozygous samples in all 20 genomic DNA samples. Overall this demonstrates the versatility of the EXPRESS SYBR® GreenER™ qPCR SuperMix, which performs reliably in sensitive qPCR reactions and HRM gene scanning applications.

References

1. Wittwer, C.T. et al. (2003) High-resolution genotyping by amplicon melting analysis using LCGreen. *Clin Chem* 49(6 pt 1):853–860.
2. Wojdacz, T.K. and Dobrovic, A. (2007) Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. *Nucleic Acids Res* 35(6):e41.
3. Liew, M. et al. (2004) Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. *Clin Chem* 50(7):1156–1164.

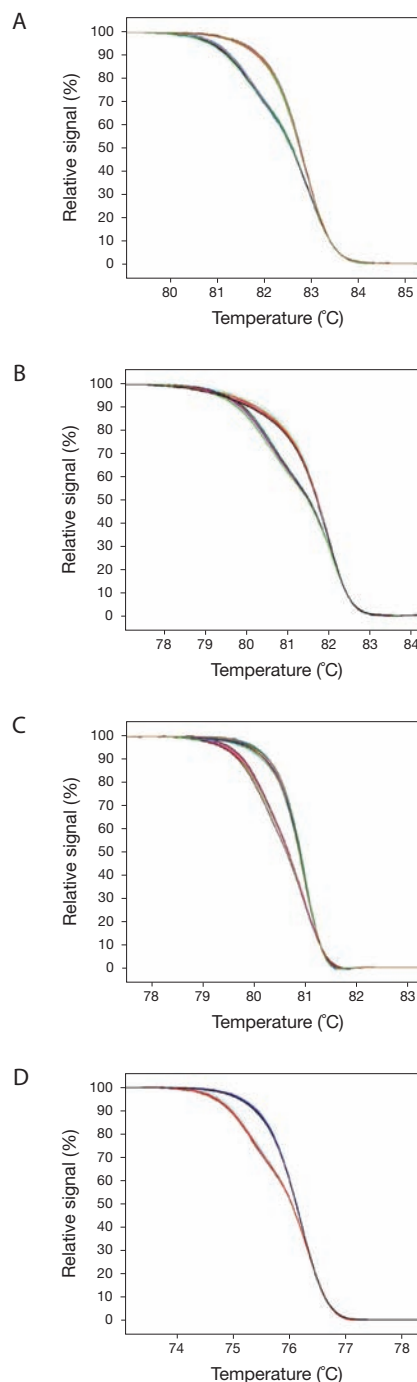


Figure 1—Fluorescence-normalized and temperature-shifted melt curves. The LightCycler® 480 Gene Scanning Software (Roche Applied Science) was used to analyze the melting profiles obtained for each type of SNP given in Table 1. The software analyzes these differences by first normalizing the fluorescence data and then temperature-shifting the curves. The panels depict as follows: A) ABCC8, SNP class 1, A/G mutation; B) CYP19A1, SNP class 2, C/G mutation; C) UGT1A1, SNP class 3, G/T mutation; D) ABCC1, SNP class 4, A/T mutation.

Ordering Information

Product	Quantity	Cat. no.
EXPRESS SYBR® GreenER™ qPCR SuperMix Universal	200 rxns	11784-200
EXPRESS SYBR® GreenER™ qPCR SuperMix Universal	5 x 200 rxns	11784-01K

Learn more about other uses for EXPRESS SYBR® GreenER™ qPCR SuperMix at www.invitrogen.com/express.



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