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High resolution melt (HRM) analysis for mutation screening of genes related to hereditary hemorrhagic telangiectasia

Comparison of Applied Biosystems® ViiA™ 7 and 7500 Fast Real-Time PCR Instruments

HRM: How it works

HRM analysis starts with PCR amplification of the region of interest in the presence of a fluorescent dsDNA-binding dye. The dye exhibits high fluorescence when bound to dsDNA and low fluorescence in the unbound state. Following PCR, the product is gradually melted using instrumentation capable of capturing a large number of fluorescent data points per °C change in temperature, with high precision. When the dsDNA dissociates (or melts) into single strands, the dye is released, causing a change in fluorescence. Fluorescence measurements are plotted to create a melt curve (or profile).

Amplicon properties such as GC content, length, sequence, and heterozygosity contribute to the shape of each amplicon's melt profile. Comparing melt profiles can provide valuable information for mutation screening, genotyping, methylation analysis, and other investigative applications.

To learn more about performing HRM experiments or to download the Applied Biosystems® HRM Getting Started Guide and Guide to High Resolution Melting (HRM) Analysis, go to www.appliedbiosystems.com/hrm.

Collaborator: Dr Sylvie Patri works in the laboratory of Génétique Cellulaire et Moléculaire du Centre hospitalier universitaire (CHU) de Poitiers, France, headed by Professor Alain Kitzis. The laboratory is mainly focused on human genetic diseases, including cystic fibrosis, hereditary hemorrhagic telangiectasia, Friedreich ataxia, Huntington disease, cerebellum ataxia, and others. They are also involved in developing and implementing molecular biology methods for mutation detection.

Multiple genetic variants can cause HHT, making HRM analysis a good candidate for its detection

Hereditary hemorrhagic telangiectasia (HHT), also known as Osler-Rendu-Weber disease, is a genetic disorder that causes arteriovenous malformations (AVMs), which can lead to frequent nosebleeds, red markings on the skin called telangiectasias, and bleeding in major organs such as the brain, liver, and intestines. HHT is an autosomal dominant disease caused by mutations in two major genes. HHT1 is associated with mutations in the endoglin gene (ENG, MIM# 131195) found on chromosome 9 [1]. Endoglin is an accessory transforming growth factor (TGF)-beta receptor. HHT2 is caused by mutations in the gene for activin receptor-like kinase type 1 (ALK1), (gene symbol ACVRL1, MIM# 601284) found on chromosome 12 [2]. ALK1 is a TGF-beta type I receptor. Both endoglin and ALK1 are involved in the TGF-beta signaling pathway. Thus far, 163 mutations of the endoglin gene and 131 mutations of the ALK1 gene have been described and, with few exceptions, these tend to be family-specific [3–7]. Researchers are interested in identifying mutations that cause HHT to help understand how critical regions of these genes contribute to the disease process. The identification of these mutations is therefore of great importance in clinical research.

High resolution melt (HRM) analysis is a rapid, cost-efficient method for identifying genetic variation without requiring specific sequence information. Since hundreds of individual nucleotide substitutions at different positions are associated with HHT1 and HHT2, HRM analysis is well-suited for screening DNA samples for the mutations that cause the disorder. In this study, Dr. Sylvie Patri and colleagues in the laboratory of Génétique Cellulaire et Moléculaire (CHU de Poitiers, France) evaluated the performance of HRM analysis using the Applied Biosystems® ViiA™ 7 and 7500 Fast Real-Time PCR Systems to detect mutations in the endoglin and ALK1 genes.

Experiment design

DNA isolation and quantification

In this study, 20 DNA samples from normal control individuals and people with previously characterized genetic variation in exons 2, 4, and 8 of the endoglin gene and exons 2, 8, and 9 of the ALK1 gene were evaluated. DNA extraction was performed using a magnetic bead–based method with the manufacturer's recommended protocol (Bionobis Magtration system 12GC sytem). DNA concentration was determined using small-volume spectrophotometry (Thermo Scientific).

Primer design

PCR primers were designed to amplify each exon in the endoglin and ALK1 genes, and their flanking regions. However, in the experiments described here, only the highlighted exons were evaluated by HRM analysis. Amplicon length was kept short for maximum genotype discrimination. For the larger exons, PCR primers were designed for two or three overlapping amplicons, to help assure complete gene coverage (Tables 1, 2). For more information on the protocol and primers, please contact Dr. Patri at s.patri@chu-poitiers.fr.

Real-Time PCR and HRM analysis

Amplification reactions (20 µL) were assembled using the PCR primers described in Table 2 and MeltDoctor™ HRM Master Mix, following the instructions provided with the MeltDoctor™ mix (Table 3). PCR thermal cycling and HRM conditions are shown in Table 4. All reactions were run in duplicate on both an Applied Biosystems® ViiA™ 7 and 7500 Fast Real-Time PCR System. After 40 cyles of PCR, reaction products were denatured at 95°C and renatured at 60°C for 1 min, and then the high resolution melt consisted of a slow denaturation from 60°C to 95°C.

Table 1. Amplicon design for analysis of the endoglin and ALK1 genes. Primers were designed for analysis of the indicated exons in the endoglin and ALK1 genes. For most exons, primer design supports HRM analysis, but for exons 3 and 10, primers were designed for analysis by sequencing.

Endoglin gene			ALK1 gene	
Exon	**Exon size (bases)**	**Amplicon size (bp)**	**Exon size**	**Amplicon size (bp)**
1	417 (coding 47)	237	Non coding	
2	152	275	66	198
3	141	241	252	Sequencing
4	163	286	212	212 + 166
5	166	259	100	242
6	127	289	147	287
7	175	311	276	182+179+144
8	143	271	198	213+195
9	138	274	131	180+195
10	39	169	311	Sequencing
11	117	266		
12	258	403		
13	55	207		
14	1,037 (coding 137)	272		

Table 2. Primer sequences.

Endoglin gene	
Exon 2	Fwd: 5′ GTAGGAGTCATTGTCATCACC 3′
	Rev: 5′ TCACCCCATCTGCCTTGGA 3′
Exon 4	Fwd: 5′ TACATGGGATAGAGAGGGCA 3′
	Rev : 5′ GGAGCTCAGATTCCTCTG 3′
Exon 8	Fwd: 5′ GCCTGGTGCGGGCACAC 3′
	Rev : 5′ GGGCTAGGGGAGGAACCA 3′
ALK1 gene	
Exon 2	Fwd: 5′ AGCCACGGCCAGCGGCT 3′
	Rev : 5′ TGATCTGGGCCCAGATAGC 3′
Exon 8	Fwd: 5′ CCCCCTGGATCCCAGGTT 3′
	Rev : 5′ CCAAAGGCCCAGATGTCAG 3′
	Fwd: 5′ CAGATCCGCACGGACTGC 3′
	Rev : 5′ CTGCAAACCTCCCAGGCC 3′
Exon 9	Fwd: 5′ GGGTGGTATTGGGCCTCC 3′
	Rev : 5′ CCAGCCGGTTAGGGATGG 3′
	Fwd: 5′ AGGACATGAAGAAGGTGGTG 3′
	Rev : 5′ GCCCTAACCAGGACACTCA 3′

HRM detects all variants—on both real-time PCR systems

In these experiments, all of the known genetic variants were clearly distinguished from one another and from wild type DNA using HRM analysis. This 100% mutation detection rate was achieved on both the ViiA™ 7 and 7500 Fast Real-Time PCR Systems. Figure 1 shows a comparison of the HRM analysis data for ALK1 exon 9 from both instruments. This difference plot analysis easily identified three variants: a wild type (WT) cluster: c. 1377+45 T>T; variant A: c.1377+45 T>C; variant B: c.1377+45 C>C; and variant C: c.1328 G>A.

Table 3. Reaction setup.

Reagents (initial concentration)	Volume/reaction (final concentration)
MeltDoctor™ HRM Master Mix (2X)	10 µL (1X)
PCR primers (forward and reverse) (2 µM)	3 µL (300 nM)
Water	4 µL
DNA (10 ng /µL)	3 µL (30 ng)
Final volume	**20 µL**

Table 4. PCR thermal cycling and HRM conditions.

Stage	Step	Temperature	Time	Ramp rate
Holding	Enzyme activation	95°C	10 min	100%
40 cycles Water	Denaturation	95°C	15 sec	100%
	Annealing/extension	60°C	1 min	100%
Melt Curve Dissociation	Denaturation	95°C	10 sec	100%
	Annealing	60°C	1 min	100%
	HRM	95°C	15 sec	0.025°C/sec
	Reannealing	60°C	15 sec	100%

ViiA™ 7 Real-Time PCR System features

Block configurations	96-well, Fast 96-well, 384-well (runs Fast or standard), TaqMan® Array Microfluidic Cards
Run time	30 min expected (Fast 96-well); 35 min (384-well)
Resolution	1.5-fold changes for singleplex reaction
Excitation source	OptiFlex™ System with halogen lamp
Detection channels	Decoupled—6 emission, 6 excitation
21 CFR p11 compliance module	Optional software module
Remote monitoring	Available to monitor up to 4 instruments in real time and the status of up to 15 instruments
Data export format	User-configurable: *.xls, * .xlsx, *.txt, and 7900 formats, as well as the new MIQE-compliant RDML format

Comparable HRM performance and results on the ViiA™ 7 and 7500 Fast systems

The results from these experiments indicate that the Applied Biosystems® ViiA™ 7 Real-Time PCR System produces results comparable to those from the 7500 Fast Real-Time PCR System. The overall HRM performance of the two platforms were also found to be comparable in this study. However, the ViiA™ 7 System, with its integrated HRM Software offered benefits in terms of ease of use and HRM-feature set:

- Single integrated HRM software for reaction analysis—on the ViiA™ 7 system, you can view and analyze the amplification plot and HRM data

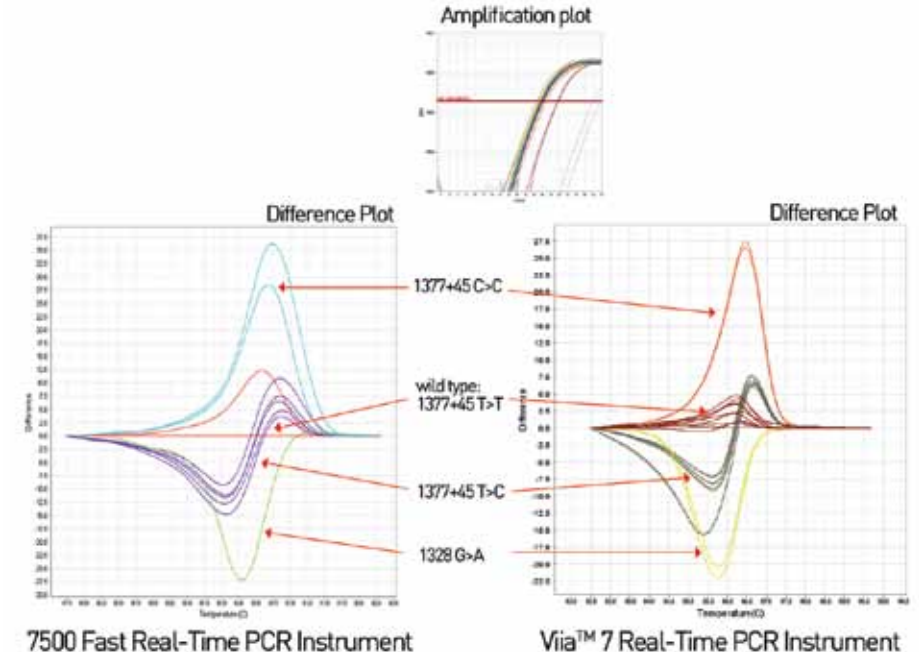

Figure 1. Clear ALK1 exon 9 variant detection on both the Applied Biosystems® ViiA™ 7 and 7500 Fast Real-Time PCR Systems.

from your experiment in the same software program. In addition, the software includes advanced features for data interpretation, such as the option to define the number of variant calls in the analysis settings.
- Enhanced instrument controls specifically designed for HRM analysis—when setting up HRM analysis on the ViiA™ 7 system, you can specify the number of data points collected per °C during the HRM, and the ramp rate setup is expressed in °C/sec.

In these HRM experiments, the ViiA™ 7 system was compared to the 7500 Fast system running HRM software v2.0. Note that HRM Software v3.0 is available for the 7500 Fast system, and it makes HRM analysis on the 7500 Fast system more convenient. Its benefits include more flexible plate layout controls and visualization, the ability to copy and paste plate setup information from Excel software, and an advanced assay settings library for assays that you run routinely. For data analysis, HRM Software v3.0 makes it easier to assign samples and select controls, allows users to specify the number of expected clusters for easier genotyping, and includes enhanced data-plot visualization tools.

HRM screening for HHT-related genetic variation on the ViiA™ 7 system is very effective

HHT research studies would benefit greatly from a rapid, simple, and accurate method for identifying DNA samples with mutations in the ALK1 and endoglin genes. Efforts to identify such a screening technique are faced with the following challenges that make screening for mutations time-consuming and costly: the lack of highly recurrent mutations in ALK1 and endoglin genes, locus heterogeneity in these genes, and the presence of mutations in almost all coding exons of the two genes.

This study confirms that HRM analysis performed on the Applied Biosystems® ViiA™ 7 Real-Time PCR System overcomes these challenges and constitutes an ideal HHT pre-screening method. Results obtained with the ViiA™ 7 and 7500 Fast Real-Time PCR Systems were comparable in this study, However, the innovative ViiA™ 7 system interface made it easy to detect genetic variants.

In this study HRM proved to be highly sensitive, rapid, specific, and reproducible. This efficient, easy-to-use HRM screening workflow may provide benefits to investigators operating in a clinical research environment.

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