High Resolution Melting Analysis (HRMA) Combining Scanning and Genotyping for Rapid Detection of Medium Chain Acyl-CoA Dehydrogenase (MCAD) Deficiency

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INTRODUCTION

MCAD deficiency is an inborn metabolic disorder with an incidence of up to 1 in 10,000 births and can result in death or serious disability. These outcomes can be prevented via diet modification by early diagnosis. The presence of a mutation c.985A>G in *ACADM* exon 11 has been linked to affected phenotype in clinical cases[1]. Additional studies have shown the prevalence of this mutation in populations other than Caucasian is low[1]. A large newborn screening study performed by the state of New York showed that affected individuals were less likely to be homozygous for the c.985A>G mutation than they were to have other types of mutations such as large deletions or nonsense mutations[2]. Another large phenotype-genotype study revealed many genetic variants of unknown significance as well as a mutation, c.199T>C in exon 3, that was present in individuals who expressed a milder form of the disease[3]. These findings suggest that genotype confirmation of MCAD deficiency cannot be limited to detection of a single mutation, and a DNA scanning technique would be useful to rapidly canvass the content of the *ACADM* gene. Gene scanning by PCR amplification and HRMA is a cost and time efficient alternative to sequencing for identifying sequence variants. Here we present a scanning panel for all 12 exons of the *ACADM* gene followed by small amplicon genotyping assays for confirmation of two point mutations that have been associated with clinical disease. Since normal variants are known to exist at high frequency in both exon 3 and 11, we have designed genotyping assays for their identification. Sequence variations detected by scanning in exons 3 or 11 would be reflex tested for both known common variants and clinically relevant variants to enable a rapid identification of clinically relevant mutations without the need for sequencing confirmation.

MATERIALS AND METHODS

Scanning PCR primers used to amplify the 12 MCAD exons were derived from designs described by McKinney *et al.*[4] and in-house designs from ARUP and Canon U.S. Life Sciences, Inc. laboratories (Table 1). Confirmation of known clinically relevant mutations was performed by reflex testing using small amplicon assays. Assays were designed for the most common pathogenic mutation (c.985A>G) and one suspected mild mutation (c.199T>C) that has been detected through newborn screening. In addition, small amplicon assays for two common variants (c.216+10T>C and c.1161A>G) were used (Table 1).

PCR and HRMA were performed using the LightScanner® 32 System with conditions shown in Table 2. Scanning assays for all 12 exons along with 4 genotyping assays were tested against 14 samples from ARUP Laboratories and 15 samples from the Coriell Institute for Medical Research, for a total of 29 clinical specimens (Table 3). Genomic DNA from the 14 ARUP samples was whole genome amplified (WGA) using the Qiagen REPLI-g® Mini kit for 2.5µl template DNA to ensure enough DNA was available for replicate analysis. Samples from Coriell were not whole genome amplified. All samples were run twice with each assay (13 Scanning, 4 Genotyping), and one no template control was used for each run.

Sequence confirmation was performed on representative samples within a HRMA cluster to validate the result if previous sequence verification on the source DNA had not been performed.

Table 1. Primer designs for scanning and genotyping assays; highlighted in blue designed by ARUP laboratories, highlighted in gray designed by Canon U.S. Life Sciences, Inc., and highlighted in white derived from McKinney *et al.*[4].

Assay	F Primer Sequence	R Primer Sequence	Amplicon Length
Exon 1	GACCCGTGTATTATTGTCCGAG	TGCTCCGACACCACAATACC	131
Exon 2	CAGTAGTCTCTTATCTGATTAATGTTTAACTTATCAAATT	AAAGCTTCATATGTATAAGTTTAAAGTCAAAAGATAGAAAC	205
Exon 3	CCTTGTTATCCAGTTTTAACTTTCTAAATAATTTTC	CAGATAGTTTGATTACATAATCTTGTAAAAAATGT	212
Exon 4	CATTTTTTACAAGATTATGTAATCAAACTATCTGGATTTCAA	GAGTTCCACAATTTTTCTTACTCATATGCATTCCAG	205
Exon 5	ATAGTTTACCTTTATTTCTATTGTGATGTACTACATATT	TTCAGGAGTAACTATCTCATTAACAAGAGC	233
Exon 6	GCATCTCTGAATTTACATATGCAAT	AAGTGTGAAATAAAGCGGCA	181
Exon 7	CATTAATTTCATTTCTCTTGTTTTTA	AGAAAAAATATACAAAGATGTTTGA	203
Exon 8	GAGCAATCACCATGTGTTAT	ATGTTTTTAATTAAAGGAAAGTTAAGTAATTT	258
Exon 9	TGATCCCTGTTTTAGGTAATTGC	GAGAAACACACTGAACATACAATTTT	342
Exon 10	ATAGACACTTAGGCAGATATTGTG	AAATTGATTAGTTTGTGGTTTAAAAATCAT	264
Exon 11_1	ACTTTTAAGTTTTCTCAATAAATATCCTTTAAT	TATCTCCAGCAAATGCCTTT	198
Exon 11_2	GTCGAAATACCTATTATGCTTCT	ATATCTCTCTCCTTGCAAAC	197
Exon 12	AAAGATATTTAACCTACACTTATATTTTTC	ACAGTGGCTTGTGTTCT	154
c.199T>C	GAAATCATCCCAGTGGCT	ACCTACTTCACCAGTTTTATCA	48
c.216+10T>C	ATGATAAAACTGGTGAAGTAGGGTA	AAAGATTTTCCCTCTTTAAAATGT	52
c.985A>G	CTGGCTGAAATGGCAATG	CTCTGGTAACTCATTCTAGC	50
c.1161A>G	GGCAATGGATTTAATACAGA	GCATCCCTCATTAGTTTTTC	50

Table 3. DNA from the Coriell Institute for Medical Research.

NA01954
NA07523
NA08684
NA11319
NA14439
NA14448
NA14501
NA17137
NA09820
NA07441
NA07552
NA08188
NA11282
NA11284
NA11254

Table 2. PCR and HRM Conditions; a) Reagent concentrations in the master mix, b) PCR Protocol, c) HRM protocol.

a) Master Mix

Reagent	Concentration
dNTPs	0.37 mM
Mg^{2+}	3 mM
F Primer	1 µM
R Primer	1 µM
LCGreen® Plus+	1X
Taq Polymerase	0.05 U/µL
Template DNA	50 ng per reaction
BSA	250 µg/mL

b) PCR Protocol

Hot Start	95°C 2 min	
Denaturation	**95°C 5 sec**	**Cycles**
Annealing	**68°C 10 sec**	35 for scanning assays
Extension	**72°C 15 sec**	40 for genotyping assays
Post-PCR	95°C 10 sec	
	45°C 10 sec	

c) HRM Protocol

"High-res melt" Data Collection
65°C → 95°C at 0.3°C/second

RESULTS

Scanning assay results produced expected patterns for HRMA in all 29 specimens tested demonstrating the ability of the assay to detect variants in all exons tested. Results are presented as difference plots and are grouped according to DNA source used for the assay, WGA or genomic. Samples used as difference plot baselines were chosen for each assay from those that fell in the middle of the normal cluster pattern. Scan results for genomic DNA samples and those amplified by WGA are shown separately since these two sample types will display different normal patterns, though variants are easily distinguishable from the normal population in most cases. Known benign variants in Exons 1, 2, 4, 7, 12 that were located in the primer binding region or outside of it showed no differences as expected.

RESULTS (continued)

Variants were detected in all other exons. In exons where both WGA and genomic samples contained the same variant, the variant was easily identified in both sample types (Figure 1, exons 3, 5, 11). Variants that were represented in only a single sample type were also distinguishable from the normal population with two exceptions. The amplified region of exon 9 appears to contain three melt domains which did not generate reproducible melt patterns, and the heterozygous samples can fall within the cluster of normal population sequences (Figure 1, exon 9). A similar case was observed with the first amplified domain of exon 11 (Figure 1 , exon 11_1). These two amplification targets would need to be redesigned to assure that a consistent difference in pattern could be detected . Variation in amplification of normal sequences created a different pattern on replicate assay runs in exon 7 (Figure 2). While the overall difference plots appeared dissimilar, the extent of overall difference (10 units) remained the same. This occurred only in this exon and no difference in assay buffer or conditions were identified to explain this.

Samples with variants identified in the scanning of exons 3 and 11 were reflex tested by small amplicon genotyping to distinguish if the variants were clinically relevant (Figure 3). Samples without variants were included in the assay for reference. All samples were correctly identified by reflex testing.

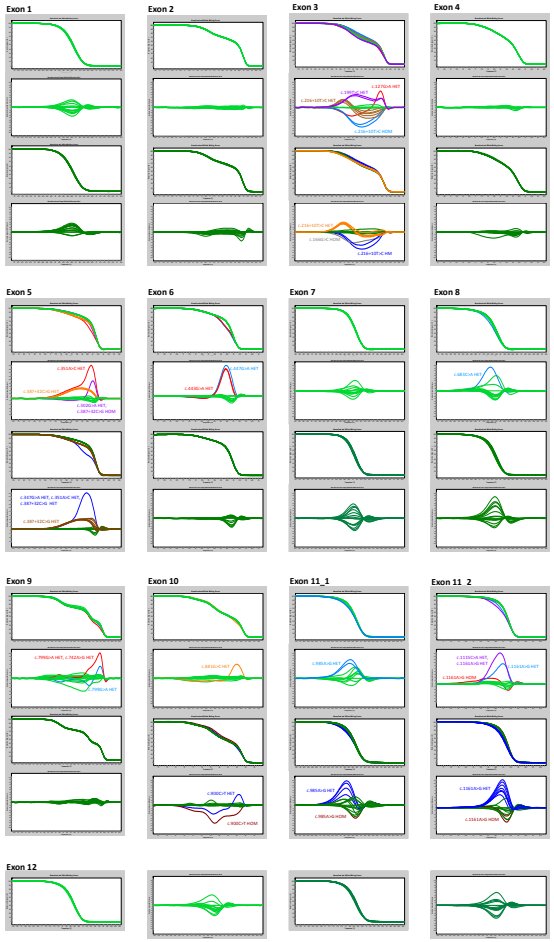

Figure 1. Images of the scanning assays for all exons (12 exons, 13 assays total). Melt curves and difference plots are shown for whole genome amplified samples and genomic DNA samples separately for each exon. Whole genome amplified samples that appear normal in any given assay are colored light green; genomic DNA samples that appear normal in any given assay are colored dark green. All other variants are labeled with a matching color in the relevant plot.

RESULTS (continued)

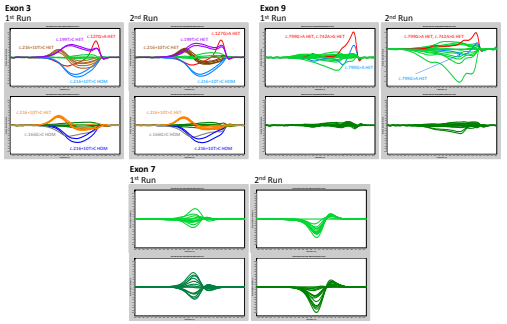

Figure 2. Reproducibility examples.

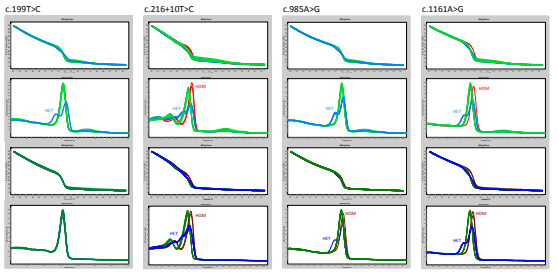

Figure 3. Images of the small amplicon genotyping assays for four targets: c.199T>C, c.216+10T>C, c.985A>G, and c.1161A>G. Melt curves and melt derivatives are shown for whole genome amplified samples and genomic DNA samples separately for each assay. Whole genome amplified samples that appear normal in any given assay are colored light green; genomic DNA samples that appear normal in any given assay are colored dark green. All other variants are labeled with a matching color in the relevant plot. The c.216+10T>C assay has an additional melt feature at a lower Tm for all DNAs. A smaller product was seen on a gel run with these PCR products in addition to the expected product, which may explain this extra feature. This assay should be redesigned.

CONCLUSIONS

We have demonstrated that gene scanning using HRMA is an effective rapid method for identifying variants in the *ACADM* gene. Thorough testing of amplification target is essential to be able to establish reliable patterns for the observed variation in the normal population and to demonstrate that variants can be distinguished. Use of WGA DNA can be invaluable during assay development since it shows similar ability in performance to genomic DNA at distinguishing variants from the normal population. However, WGA samples cannot be used to define HRMA patterns that would be used to evaluate genomic DNA. Identification of baseline sequences that produce consistent patterns and defining the normal spread of difference across normal sequences are crucial to validation of HRMA scanning methods. Sequence confirmation of mutation scanning can take several days to move through testing and data review procedures. Rapid turnaround of confirmation testing by HRMA genotyping assays from positive screening results for errors of inborn metabolism such as MCAD would allow for early dietary intervention. Using genotyping to distinguish between common variants and clinically relevant mutations by HRMA rather than by sequencing allows the entire test cycle to be completed within a single laboratory shift.

REFERENCES

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