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Two-dye based arrayed primer extension for simultaneous multigene detection in lipid metabolism ☆

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ABSTRACT

Background: Cardiovascular disease (CVD) is one of the major causes of death worldwide. Numerous genetic risk factors in lipid metabolism, including mutations of *LDLR*, *APOB*, and *PCSK9*, as well as polymorphisms of *CETP* and *APOE*, have been found to associate with CVD.
Methods: In this study, a two-dye based arrayed primer extension (APEX) microarray assay for simultaneous multigene (*LDLR*, *APOB*, *PCSK9*, *CETP*, and *APOE*) detection was developed. The DNA templates, originating from 1 DNA sample of known genotype and 7 blind DNA samples, were amplified by uniplex PCR.
Results: Optimized conditions for the APEX reaction were determined to include a hybridization temperature of 55 °C and a DNA template size of 50–150 bp. The total assay including PCR, purification, fragmentation, APEX reaction, and image analysis could be performed in 6 h. In total, 48 genotypes were identified among 8 individual DNA samples by APEX analysis.
Conclusions: The data suggest that this APEX microarray offers a robust, fast, and versatile option for screening these genotypes in hypercholesterolemia patients.

1. Introduction

Cardiovascular disease (CVD) is the number one cause of death globally [1]. WHO reported that approximately 17.1 million people died from CVD in 2004, representing 29% of all global death, and that almost 23.6 million people will die from CVD by 2030 [1]. CVD was also verified as one of the major causes of death in Thailand [2]. Risk factors include those that are controllable, such as smoking, diet, and physical activity, and those that are uncontrollable, such as the genetic background of genes involved in lipid metabolism. Examples of genes that have been associated with CVD include *LDLR*, *APOB*, *PCSK9*, *CETP*, and *APOE* [3–5].

Mutations in the *LDLR* gene result in familial hypercholesterolemia (FH; OMIM #143890), an autosomal dominant disorder with mutations occurring at a frequency of 1:500 for heterozygotes and 1:1,000,000 for homozygotes in the general population [6]. Previous studies have reported two *LDLR* mutations, D151Y and M391T in Thai FH patients [7,8]. Recently, M391T was identified in a female Chinese Thai FH patient [9]. Apart from FH, a similar phenotype was found in familial defective apolipoprotein B-100 (FDB; OMIM #144010), an autosomal co-dominant disorder, which is caused by the mutation of *APOB* gene. The most common *APOB* mutation is R3500Q [10]. As well, mutations in a third locus, pro-protein convertase subtilisin/kexin type 9 (PCSK9) (OMIM#603776; #607786), have been reported to result in hypercholesterolemia. The D374Y mutation in *PCSK9* results in an especially severe clinical phenotype [11,12]. Furthermore, the ε4 allele of *APOE* has been found to associate with increased levels of cholesterol and to be a risk factor for both heart and Alzheimer's disease [13,14], whereas the *Taq*IB polymorphism of *CETP*, the B1 allele, has been found to associate with both higher CETP concentration/activity and lower levels of HDL cholesterol [15].

Several molecular methods were developed to screen for mutations in hypercholesterolemia patients, such as single-strand conformation polymorphism (SSCP) analysis [16], denaturing gradient gel electrophoresis (DGGE) [17], denaturing high performance liquid chromatography

Abbreviations: APEX, arrayed primer extension; apoB-100, apolipoprotein B-100; apoE, apolipoprotein E; CETP, cholesteryl ester transfer protein; CVDs, cardiovascular diseases; DGGE, denaturing gradient gel electrophoresis; DHPLC, denaturing high performance liquid chromatography; FH, familial hypercholesterolemia; HDL, high density lipoprotein; LDLR, low density lipoprotein receptor; meltMADGE, melt-microplate array diagonal gel electrophoresis; PBS, phosphate buffer saline; PCR, polymerase chain reaction; PCSK9, proprotein convertase subtilisin/kexin type 9; PMT, photo multiplying tube; SSCP, single-strand conformation polymorphism.

☆ Disclosure: The authors report no conflicts of interest.

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Table 1
Oligonucleotide probes spotted on microarray for detection mutations and polymorphisms.

Gene	Nucleotide change	Amino acid change	Tm (°C)	Probes (25–34 bp) [a]	
LDLR	G514T	D151Y	69.5	S	5′**TT**TGTGGGCCTGCGACAACGACCCC 3′
			66.3	AS	3′TGACGCTTCTACCGAGCCTACTCAC 5′
	T1235C	M391T	67.9	S	5′ACCAACCGGCACGAGGTCAGGAAG A3′
			67.9	AS	3′CTGCGACCTGGCCTCGCTCATGT**TT** 5′
APOB	G10708A	R3500Q	65.4	S	5′CCAACACTTACTTACTTGAATTCCAAGAGCACAC 3′
			66.3	AS	3′CAGAAGTCACTTCGACGTCCCGTGA 5′
PCSK9	G17908T	D374Y	67.9	S	5′ GGAGG ACATCATTGG TGCCTCCAGC 3′
			64.6	AS	3′ TGACGTCGT GGACGAAACA CAGTGT 5′
CETP	G + 279A	–	67.9	S	5′CTGCGACCCAGAATCACTGGGGTTC 3′
	(TaqIB)		64.6	AS	3′TCAATCCCAAGTCTAGACTCGGTCC 5′
APOE	ε2	C112	69.5	S	5′**TT**TGGGCGCGGACATGGAGGACGTG3′
	112T, 158T	C158	69.5	AS	3′CGCCGGCGGACCACGTCATGG**TTTT** 5′
	ε3	C112	69.5	S	5′CCGCGATGCCGATGACCTGCAGAAG 3′
	112T, 158C	R158			
	ε4	R112	69.5	AS	3′CGGACCGTCACATGGTCCGGCC**TTT** 5′
	112C, 158C	R158			

A = Sense strand
AS = Anti-sense strand
[a] Oligonucleotide probes were modified at 5′ end with 6-carbon amino linkers. Some strands were modified by adding artificial T (represented with bold letter) at 5′ end in order to adjust a similar of melting temperature.

(DHPLC) [18], a SSCP/heteroduplex capillary electrophoresis method [19], and melt-microplate array diagonal gel electrophoresis (meltMADGE) [20]. However, these methods are time consuming and labor intensive. To overcome these challenges, cost-effective and efficient high-throughput technologies, such as NanoChip microelectronic arrays [21], and oligonucleotide microarrays [22], were recently developed. However, both of these latter techniques can only screen a limited type and number of genetic mutations. The arrayed primer extension (APEX) microarray [23], which takes advantage of Sanger dideoxy sequencing methodology, is another fascinating technique to be developed to gain more flexibility in high-throughput genetic analysis. This work is the first use of two-dye based arrayed primer extension (APEX) microarrays for screening *LDLR*, *APOB*, *PCSK9* mutations, as well as *APOE* and *CETP* polymorphisms.

2. Materials and methods

2.1. DNA samples

A DNA sample of known genotype, which was identified by directed DNA sequencing, and genomic DNA from 7 blind specimens were included in this study. Among 7 blind samples, 3 samples were positive for the *LDLR* M391T, *APOB* R3500Q, and *PCSK9* D374Y mutations. The

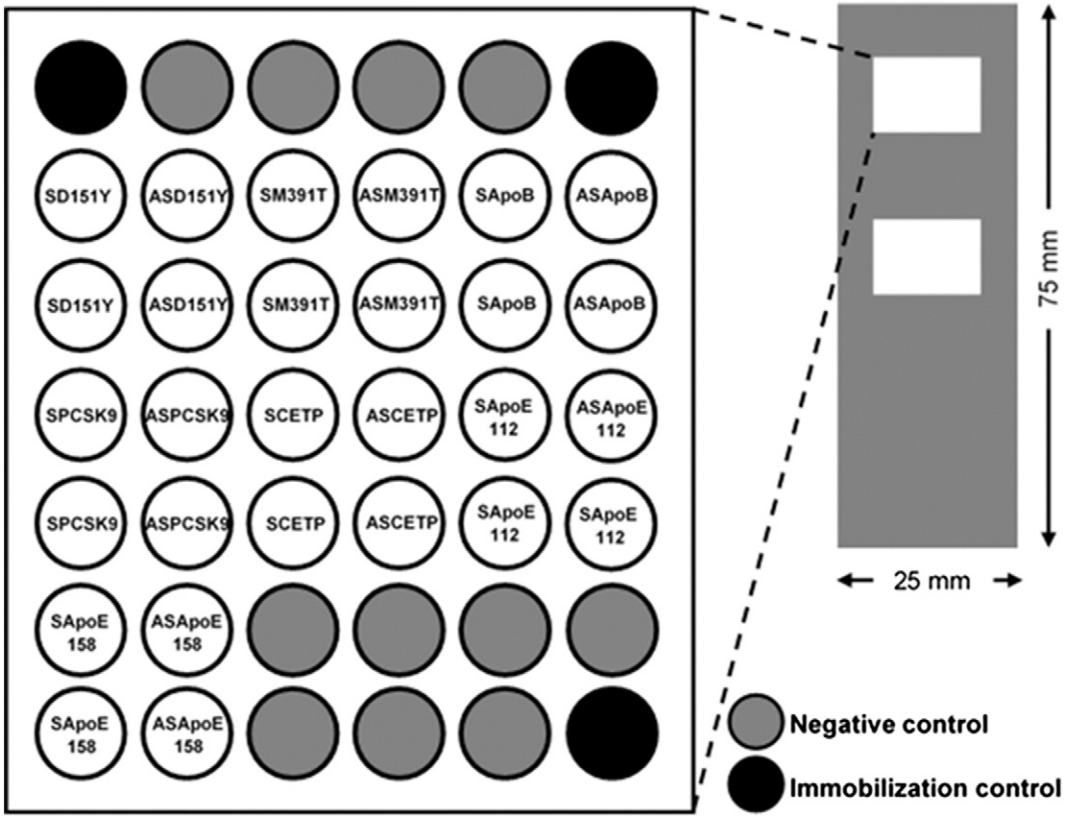

Fig. 1. The microarray layout. The slide was divided into two hybridization areas. Each hybridization area contained the probes of sense (S) and antisense (AS) that were spotted in duplicate in the following arrangement; negative control, and immobilization control. The array layout was set to 6 × 7 spots per subarray.