OPTIMIZATION OF PCR-MULTIPLEX SSCP TO IDENTIFY MUTATIONS AND POLYMORPHISMS AT THE LDL RECEPTOR LOCUS

PATTHAMAWADEE CHAROENSUK

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ABSTRACT

Familial Hypercholesterolemia is caused by a mutation within the low density lipoprotein receptor (LDLR) gene. The mutation impairs proper function of LDLR and results in very high level of plasma cholesterol. Such levels result in early and severe atherosclerosis and hence substantial excess mortality from coronary heart disease. Diagnosing FH on clinical grounds is relatively difficult, and previous genetic methods are too cumbersome for routine use. Thus, there is a need to develop screening strategies to ensure that more patients are being correctly diagnosed and treated. In this study, restriction enzymes were used to digest hypermutable CG region of some PCR-amplified exons to increase the sensitivity of multiplex SSCP. These RE digested-PCR products were selectively combined and analyzed by multiplex SSCP. Moreover, the multiplex SSCP was analyzed in gradient polyacrylamide gel to increase the resolution. The conditions of these multiplex-SSCP analyses were optimized until individual exon of each multiplex set was separately apparent. Six combination sets of PCR-multiplex SSCP were set up and five of them were validated by known mutations (M412T, S554L and IVS3+1G>T) and known polymorphisms (G1414A). No validation in combination set 3 was made because no mutation or polymorphism was available in this combination set. The common AvaII (exon13 in combination set 4), HincII (exon12 in combination set 5) and a novel polymorphism in exon 8 (in combination set 5) were readily detected in these samples using the PCR-multiplex SSCP protocols developed in this study. These optimized PCR-multiplex SSCP protocols are expected to be useful for screening mutations and polymorphisms in the whole coding region (plus promoter) of LDL receptor gene. It was rapid, simple, sensitive and was expected to be a potential tool for mutation screening of large numbers of clinical samples.

KEY WORDS: Familial Hypercholesterolemia, LDLR, PCR-multiplex SSCP, gradient polyacrylamide gel, Novel polymorphism

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การหาสภาวะที่เหมาะสมที่สุดของเทคนิค PCR-multiplex SSCP เพื่อค้นหาการกลายพันธุ์และความหลากหลายในยีน LDL receptor (OPTIMIZATION OF PCR-MULTIPLEX SSCP TO IDENTIFY MUTATIONS AND POLYMORPHISMS AT THE LDL RECEPTOR LOCUS)

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บทคัดย่อ

Familial hypercholesterolemia (FH) เป็นโรคทางพันธุกรรมที่เกิดจากการกลายพันธุ์ในยีน low density lipoprotein receptor (LDLR) ส่งผลให้การทำหน้าที่ของ LDLR ผิดปกติไป ทำให้เกิดการเพิ่มขึ้นของระดับโคเลสตอลรอลในกระแสเลือด ระดับโคเลสตอลรอลที่เพิ่มขึ้นนี้จะทำให้เกิดกระบวนการตีบตันของหลอดเลือด (Atherosclerosis) และเสียชีวิตเพราะโรคหลอดเลือดหัวใจอุดตันในที่สุด การตรวจพบความผิดปกติของยีน LDLR ตั้งแต่เริ่มแรก (early diagnosis) จะทำให้สามารถป้องกันการเกิดโรคหลอดเลือดหัวใจได้ ปัจจุบันการวินิจฉัยโรคโดยอาศัยข้อมูลทางคลินิกเพียงอย่างเดียวนั้นยังไม่เพียงพอ จึงได้มีการวินิจฉัยยืนยัน FH ด้วยเทคนิคทาง Molecular Biology อีกด้วย อย่างไรก็ตามเทคนิคเหล่านี้ค่อนข้างยุ่งยากและใช้เวลามากสำหรับยีน LDLR ซึ่งเป็นยีนที่ค่อนข้างใหญ่ การศึกษาในครั้งนี้จึงได้พัฒนาเทคนิค PCR-multiplex SSCP ขึ้น โดยใช้เอ็นไซม์ตัดจำเพาะ ตัดตรงบริเวณที่เป็น CG (ซึ่งเกิดการกลายพันธุ์ได้ง่าย) ของชิ้นส่วนดีเอ็นเอ และ ทำ gradient polyacrylamide gel electrophoresis เพื่อเพิ่มความไวให้กับเทคนิค SSCP อีกด้วย จนกระทั่งได้สภาวะที่เหมาะสมและแยกแยะความแตกต่างได้อย่างชัดเจน จากการศึกษา สามารถจัดจำแนก PCR-multiplex SSCP ได้ 6 กลุ่ม สำหรับการวิเคราะห์ promoter และ exons ทั้งหมดของยีน LDLR และพิสูจน์ว่าวิธีนี้ใช้ได้จริงด้วยการนำตัวอย่างของผู้ป่วยที่ทราบว่ามีความผิดปกติทางพันธุกรรมหรือมีการเปลี่ยนแปลงของลำดับเบสมาทดสอบ นอกจากนี้ยังใช้ screen ผู้ป่วยจำนวน 13 คน และสามารถตรวจพบการเปลี่ยนแปลงของความหลากหลายทางพันธุกรรมชนิด AvaII และ HincII และยังค้นพบการเปลี่ยนแปลงความหลากหลายทางพันธุกรรมชนิดใหม่ใน exon 8 อีกด้วย คาดว่าวิธี PCR-multiplex SSCP นี้จะเป็นประโยชน์ในการตรวจกรองความผิดปกติของยีนหรือการเปลี่ยนแปลงลำดับเบสทั้งหมดของยีน LDLR ซึ่งคิดว่าจะเป็นวิธีที่รวดเร็ว ง่ายและมีความไวสูงเหมาะสำหรับใช้ตรวจกรองตัวอย่างที่มีปริมาณมากๆได้

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LISTS OF ABBREVIATIONS

ASO	= allele-specific oligonucleotide
bp	= base pairs
CAD	= coronary artery disease
CHD	= coronary heart disease
^{o}C	= degree celcius
dATP	= deoxyadenosine 5'-triphosphate
dCTP	= deoxycytosine 5'-triphosphate
ddNTP	= dideoxynucleotide
dGTP	= deoxyguanosine 5'-triphosphate
DGGE	= denaturing gradient gel electrophoresis
dTTP	= deoxythymidine 5'-triphosphate
DNA	= deoxyribonulceic acid
dNTP	= deoxynucleotide 5'-triphosphate
EGF	= epidermal growth factor
ER	= endoplasmic reticulum
FFAs	= free fatty acids
FH	= familial hypercholesterolemia
Fig	= figure
g	= gramme
gDNA	= genomic deoxyribonulceic acid
hr	= hour
HDL	= high density lipoprotein
HL	= hepatic lipase
HMG-CoA	= 3-hydroxy-3-methlyglutaryl-CoA reductase
IDL	= intermediate density lipoprotein
LCAT	= lecithin cholesterol acyltransferase
LDL	= low density lipoprotein
LDL-C	= low density lipoprotein cholesterol

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LISTS OF ABBREVIATIONS (Cont)

LDLR	= low density lipoprotein receptor
LPL	= lipoprotein lipase
LRP	= LDL receptor-related protein
mg	= milligramme
min	= minute
mRNA	= messenger ribonucleic acid
ml	= millilitre
mM	= millimolar
MW	= molecular weight
ng	= nanogramme
PCR-RFLP	= polymerase chain reaction- restriction fragment length polymorphisms
PCR-SSCP	= polymerase chain reaction- single strand conformation polymorphism
RE	= restriction endonuclease
pmole	= picomole
SR-BI	= scavenger receptor class BI
SREBP	= sterol-regulatory element-binding protein
SSCP	= single strand conformation polymorphism
VLDL	= very low density lipoprotein
WHO	= World Health Organization
µl	= microlitre

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CHAPTER I
INTRODUCTION

Coronary artery disease (CAD) is a multifactorial disorder resulting from the interplay of genetic and environmental factors. It is a major cause of morbidity and mortality in most Western countries. In Thailand it is also a major cause of death in our population. This multifactorial disease presents a particular challenge to different medical disciplines and is frequently associated with acquired familial hypercholesterolemia. It is clearly that the increase in plasma LDL (Low density lipoprotein) cholesterol is due to genetic alterations of LDLR (Low density lipoprotein receptor) gene specifying the formation of the LDLR, leading to defective catabolism of LDL (1). The LDLR gene spans 45 Kb on the short arm of chromosome 19 and consists of 18 exons and 17 introns. RNA splicing results in an mRNA of 5.3 Kb, with a coding region of 2.5 Kb and a protein product of 860 amino acids (2). Mutations in two other genes also cause the clinical FH phenotype. One of these is the apolipoprotein B-100 gene (APOB), located on chromosome 2p23-24, that codes for the protein component of LDL particles. In contrast to LDLR, only a small number of functional mutations have been identified in APOB. The third gene, proprotein convertase subtilisin/kexin type 9 (PCSK9), was recently identified on chromosome 1p32. To date, no epidemiologic research has investigated mutations in PCSK9.

LDLR mutations can be classified according to the effect they have on LDL receptor protein function. The LDLR protein is a cell surface receptor that removes LDL particles from the plasma by way of receptor-mediated endocytosis. In class 1 mutations, the LDLR protein is not synthesized; in class 2 mutations, the LDLR is not transported to the Golgi; in class 3 mutations, the LDLR does not properly bind with the LDL particles; in class 4 mutations, bound surface receptors are not internalized; and in class 5 mutations, the internalized LDL particles are not released in the endosome (3).In most populations, the prevalence of heterozygous FH is 1 in 500; 1 in 10^6 is found to be homozygous. Heterozygous patients carry a high risk of coronary

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artery disease or myocardial infarction in their fourth or fifth decade of life and homozygous or compound heterozygous patients in their first or second decade of life (4). Early diagnosis of at-risk individuals will allow early pharmacologic and dietary treatment with the potential of reducing the risk of CAD. Diagnosis of FH is currently based on clinical, biochemical, and genetic criteria rather than on the identification of disease-causing mutations. Molecular diagnosis of FH by the identification of disease-causing mutation is labor-intensive, time consuming, and expensive because of the large size of the LDLR gene (45kb encoding 18 exons for a 5.3 kb mRNA). In addition, there are many rare or "private" mutations, including deletions in approximately one third of patients that add to the complexity of the molecular genotype. Consequently, very few clinical laboratories offer general molecular testing for FH, and those that do limit the analysis to a subset of mutations that are prevalent in certain ethnic groups (6).

Different strategies for mutation screening in the LDLR gene have been described. Most defects have originally been detected by restriction fragment length polymorphism (RFLP) analysis, but the method limited in the detection of point mutations, small deletions or insertions. Therefore, other, more sensitive methodologies such as analysis by denaturing gradient gel electrophoresis (DGGE) or single strand conformational polymorphism (SSCP), followed by DNA sequence determination, are more appropriate (2, 5). SSCP analysis was originally described by Orita *et al.* (13). This technique is a method capable of identifying most sequence variations in a single strand of DNA, typically between 150 and 250 nucleotides in length. Under nondenaturing conditions a single strand of DNA will adopt a conformation (presumably dependent on internal base-pairing between short segments by fold back) that is uniquely dependent on its sequence composition. This conformation will usually be different if even a single base is changed. Most conformations seem to alter the physical configuration or size sufficiently that, even though the variant sequence has the same charge, the configuration-to-charge (size-to-charge) ratio is different enough to be detectable as a mobility difference upon electrophoresis through a retarding matrix such as acrylamide gel (8). The optimal length of DNA for SSCP analysis appears to be from 150 to 200 nucleotides with mutation detection sensitivity for fragments size ranging from 70-90%. Sensitivity of

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this technique decreases with increasing size of the fragment (7). Many modifications to the original protocol were developed (9-12).

At present, blood cholesterol levels tend to increase in Thai populations leading to high risk of coronary artery disease (37). This incident may be due to life style and genetic factor such as LDLR gene mutations. In case of genetic abnormality at the LDLR locus, DNA-based tests can provide unequivocal diagnosis of FH (5, 19). These DNA-based tests require a database of mutations, which are mostly ethnic specific. However, in Thai ethnic populations, database for LDLR gene mutations is not available. To establish a database of LDLR gene mutations for Thai ethnic populations, molecular analysis of the LDLR gene in Thai people should be essentially undertaken. However, molecular analysis using previous genetic methods such as PCR, PCR-RFLP, and large gel SSCP analysis are too cumbersome for routine screening (14). To avoid these problems and to obtain efficient and rapid genetic testing for all mutations in the LDLR gene, method of high sensitivity and rapid screening is essentially required. This project is thus proposed to develop such a method for identification of polymorphisms and mutations of the LDL receptor gene in population of Thai ethnic background.

In this development, restriction enzymes were used to digest hypermutable CG regions of some PCR-amplified exons to increase the sensitivity of multiplex SSCP. These RE digested-PCR products were selectively combined and analyzed by multiplex SSCP. Moreover, the multiplex SSCP was analyzed in gradient polyacrylamide gel to increase the resolution. The conditions of these multiplex-SSCP analyses were optimized until individual exon of each multiplex set was separately apparent. Therefore, abnormal mobility shift in an exon could be specified in each multiplex set and then subsequent DNA sequencing analysis could be made for that specified exon. Six combination sets of PCR-multiplex SSCP were set up and five of them were validated by known mutations (M412T, S554L and IVS3+1G>T) and known polymorphisms (G1414A). No validation in combination set 3 was made because no mutation or polymorphism was available in this combination set. These optimized multiplex-SSCP protocols were subsequently used to screen for polymorphisms and mutations in the whole coding region (plus promoter) of LDL receptor gene in a number of hypercholesterolemic patients (n=13). The common

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AvaII (exon13 in combination set 4), HincII (exon12 in combination set 5) and a novel polymorphism in exon 8 (in combination set 5) were readily detected in these samples using the PCR-multiplex SSCP protocols developed in this study. These optimized PCR-multiplex SSCP protocols are expected to be useful for screening mutations and polymorphisms in the whole coding region (plus promoter) of LDL receptor gene. It was a rapid, simple, sensitive and was expected to be a potential tool for mutation screening of large numbers of clinical samples.

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OBJECTIVES

1. To optimize multiplex SSCP method.

2. To investigate polymorphisms and mutations in whole coding region of the LDL receptor gene in Thai subjects with primary hypercholesterolemia, using the developed multiplex SSCP method.

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CHAPTER II
LITERATURE REVIEW

1. Cholesterol Metabolism

Cholesterol is an important structural component of cell membranes and a precursor molecule for the synthesis of steroid hormones, bile acids and vitamin D. The cellular requirement for cholesterol is satisfied either by *de novo* synthesis within the cell, or by being supplied from extra-cellular sources. Cholesterol accumulating within the cell above the amount capable of being utilized by the cell is esterified with a long-chain fatty acid and stored within the cytoplasm as cholesteryl ester droplets. Efflux of excess cholesterol from the peripheral tissues occurs via reverse cholesterol transport, a pathway necessary to maintain cellular cholesterol homeostasis (15).

1.1 Lipoproteins

Both *de novo* synthesized cholesterol and cholesterol derived from the diet are transported in the plasma predominantly as cholesteryl esters associated with lipoprotein particles. Lipoprotein particles are spherical with a central core of nonpolar lipids (primarily triglycerides and cholesteryl esters) and a surface monolayer of polar lipid (primarily phospholipids) and noncovalently bound apoproteins (Fig.1).

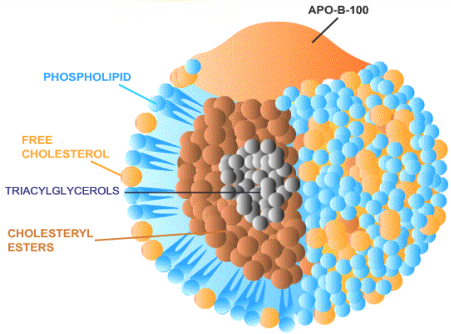

Figure 1 Low density lipoprotein structure
(http://www.med.unibs.it/~marchesi/biotec/slides/lipoproteine/LDL.gif)

Lipoproteins are classified by the type and ratio of protein and lipids that they contain, which determines their size and density (Table 1)

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Table 1 Composition of the major human plasma lipoproteins

Lipoprotein	Source	Apoliporoteins	% Protein	Core lipids %TG/%CE	Diameter (nm)	Density (g/ml)
Chilomicrons	Intestine	A(I, II, IV), B-48, C(I, II, III), E	2	86/3	75-100	<0.95
VLDL	Liver	B-100, C(I, II, III), E	8	55/12	30-80	0.93-1.006
IDL	VLDL	B-100, C(I, II, III), E	19	23/29	25-35	1.006-1.019
LDL	VLDL	B-100	22	6/42	18-25	1.019-1.063
HDL_2,HDL_3	Intestine,liver: Chylomicrons, VLDL	A(I, II, IV), C(I, II, III), D, E	40-55	3-5/13-17	5-12	1.063-1.21

1.2 Lipoproteins Metabolism

Dietary lipids are packaged in intestinal mucosal cells and secreted into the lymph as large triglyceride-rich particles called chylomicrons. Cholesterol absorption is mediated in part by the ABCG5 and ABCG8 transporters. Upon entering the circulation, lipoprotein lipase (LPL) rapidly lipolyzes the chylomicrons, thereby delivering free fatty acids (FFAs) to tissues such as muscle and adipose. The LDL (apoB/E) receptor and the LDL receptor-related protein (LRP) mediate the rapid uptake of the resulting remnant particles by liver (Fig.2) (16).

The liver actively synthesizes triglycerides and cholesterol, which, along with intestinally derived lipids, are packaged and secreted as very low-density lipoproteins (VLDLs). Like chylomicrons, VLDL particles are large and triglyceride-rich, but they have a different metabolic fate. VLDLs undergo lipolysis in the circulation and give rise to intermediate-density lipoproteins (IDL). Hepatic receptors remove a significant fraction (about half) of IDLs from the circulation, and the remainder undergo further lipolysis by LPL and hepatic lipase (HL) to produce LDLs. LDLs have a long half-life in the circulation and, in most individuals, are the predominant cholesterol-carrying particles. The LDL (apoB/E) receptor, which is

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present in both liver and peripheral tissues, largely mediates the removal of LDL from the circulation.

HDLs are formed in the circulation from precursors secreted by liver and intestine, and from chylomicron and VLDL surface remnants. HDLs facilitate the removal of cholesterol from cells, a process mediated in part by ABCA1. The cholesterol is then esterified by lecithin cholesterol acyl transferase (LCAT) and transported to the liver, the only tissue capable of metabolizing and excreting excess cholesterol. The liver's uptake of HDL cholesterol is mediated partially by the scavenger receptor class BI (SR-BI). Hepatocytes can oxidize cholesterol to bile acids by a pathway involving cholesterol 7α-hydroxylase (7-α-hydroxylase) (16).

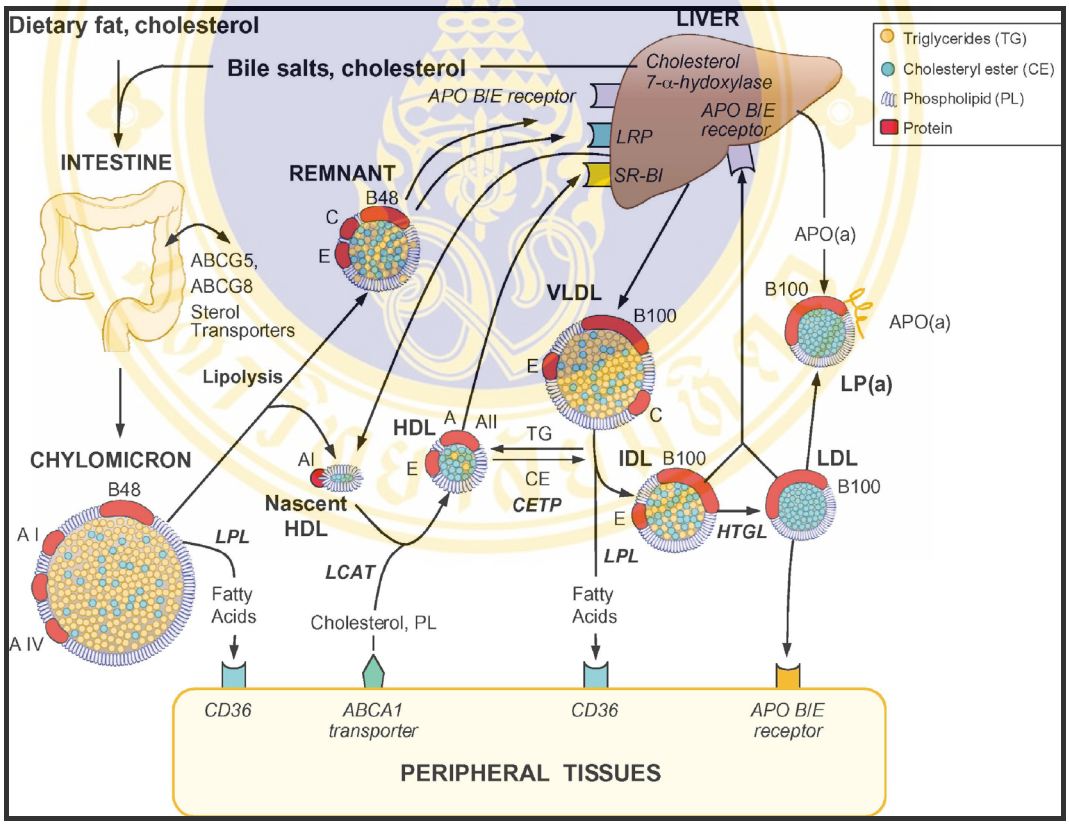

Figure 2 Lipoprotein metabolism (16)

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1.3 Lipoprotein Disorder

Lipoprotein disorders are some of the commonest metabolic diseases seen in clinical practice. Currently there is no satisfactory comprehensive classification of lipoprotein disorders. Genetic classifications have been attempted but are becoming increasingly complex as different mutations are discovered.

Table 2 Some genetic causes of dyslipidaemia (17)

Disease	Genetic defect	Fredrickson	Rick
Familial hypercholesterolaemia	Reduced numbers of functional LDL receptors	IIa or IIb	CHD
Familial hypertriglyceridaemia	Possibly single gene defect	IV or V	
Familial combined Hyperlipidaemia	Possibly single gene defect	IIa, IIb, IV or V	CHD
Lipoprotein lipase deficiency	Reduced levels of functional LPL	I	Pancreatitis
Apo C-II deficiency	Inability to synthesize apo C(cofactor for lipoprotein LPL)	I	Pancreatitis
Abetalipoproteinaemia	Inability to synthesize apo B	Normal	Fat soluble vitamin deficiencies, neurological deficit

In practice, lipoprotein disorders are simplistically classified as being:

Primary-when the disorder is not due to an identifiable underlying disease. The Fredrickson or World Health Organization classification is the most widely accepted for the primary hyperlipidaemias (Fig.3). It relies on the findings of plasma analysis, rather than genentics. As a result, patients with the same genetic defect may fall into different groups, or may change grouping as the disease progresses or is treated. The major advantage of this classification is that it is widely accepted and gives some guidance for treatment (Table 2). The six types of

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hyperlipoproteinaemia defined in the Fredrickson Classification are not equally common. Types I and V are rare, while types IIa, IIb and IV are very common.

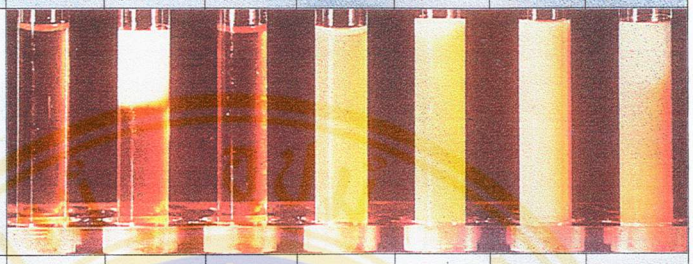
Type	Normal	Type I	Type IIa	Type IIb	Type III	Type IV	Type V
Sample							
Lipoprotein	N	↑ Chylomicrons	↑ LDL	↑ LDL ↑ VLDL	↑ IDL	↑ VLDL	↑ Chylomicrons ↑ VLDL
Total Cholesterol	N	N or ↑	↑	↑	↑	N or ↑	N or ↑
Triglycerides	N	↑↑	N	↑	↑	↑	↑↑
LDL-CHOL	N	N or ↓	↑	↑	N or ↓	N	N
HDL-CHOL	N	N or ↓	N or ↓	N or ↓	N or ↓	N or ↓	N or ↓

Figure 3 Fredrickson (WHO) classification of dyslipidaemia (17)

This classification is based on the appearance of a fasting plasma sample after standing for 12 hours at 4°C and analysis of its cholesterol and triglyceride content

Secondary- when the disorder is a manifestation of some other disease. Secondary hyperlipoproteinaemia is a well-recogniqed feature of a number of diseases which can divided broadly into two categories:

- Clinically obvious disease such as renal failure, nephritic syndrome and cirrhosis of the liver.
- Covert conditions which may present as hyperlipidaemia. There include hypothyroidism, diabetes mellitus and alcohol abuse (17).

1.4 LDL-cholesterol and atherosclerosis

The presence of LDLR and reverse (HDL) cholesterol transport pathways allows for a sensitive regulation of cellular cholesterol content throughout the body. Unfortunately, this regulation is not always flawless. Absorption of excess

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cholesterol can potentially increase the amount of cholesterol stored in the liver. This, in turn, can result in increased VLDL secretion, and subsequent LDL formation, and also down regulation of hepatic LDLR activity. Such events will potentially increase plasma LDL-cholesterol levels. Defects in the cholesterol metabolism can further induce accumulation of cholesterol in the periphery causing cardiovascular disease.

Atherosclerosis is a life-long process that begins with innocuous fatty streaks in childhood and progresses through several stages of plaque formation and gradual narrowing of the large arteries to subsequent heart attacks, strokes and peripheral vascular disease. As a major cholesterol-carrying lipoprotein in human plasma, LDL plays a major role in the development of atherosclerosis. The central process in the development of atherosclerosis is the infiltration of atherogenic cholesterol-rich lipoproteins, including LDL and VLDL, into the artery wall where subendothelial macrophages are activated by oxidized LDL particles via the scavenger receptor pathway. Total cholesterol levels of >6 mmol/l in the circulation are associated with substantially increased risk of CHD. According to the current hypotheses, a priming factor in the development of atherosclerotic plaques is endothelial dysfunction due to oxidative stress, inflammation, infectious micro-organisms, or shear stress.

Generally atherogenesis is considered a polygenic disease and numerous candidate genes are proposed. In addition to environmental factors, gene mutations affecting any of the metabolic pathways involved in the development of atherosclerosis may contribute to the risk of CHD. Not all cardiovascular diseases are polygenic in nature, and among patients with CHD onset before the age of 55, about 5% of cases are attributable to heterozygous FH (15).

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2. Familial Hypercholesterolemia

2.1 Clinical features

FH was the first genetic disease of lipid metabolism to be clinically and molecularly characterized. It is a dominantly inherited autosomal disease characterized by extremely high levels of plasma cholesterol and LDL, which contribute to the formation of cutaneous and tendon xanthomas, arcus corneae and premature cardiovascular disease. Clinically identified FH usually results from defects in the LDLR gene. Homozygous deficient patients with two abnormal LDLR genes, either identical or different mutant genes, typically exhibit life-threatening coronary atherosclerosis and subsequent myocardial infarction before age 30 (15).

2.2 Prevalence of FH

FH is the most common and most severe form of monogenic hypercholesterolemia. In most countries the prevalence of the heterozygous form of FH, in which a defective gene for the LDLR is inherited from one parent and a normal gene from another, is 1:500 and that of the homozygous form of FH is 1: 1,000,000 individuals, which renders FH probably the most common disease caused by a single-gene mutation in humans. It has been estimated that worldwide there are 10,000,000 people with FH of whom less than 10% are diagnosed, and less than 25% treated with LDL-lowering drugs. In many cases the diagnosis is missed until a dramatic clinical event occurs. In small number of genetically relatively isolated communities the prevalence of heterozygous FH is much higher than in most populations. In these populations, few LDLR mutations predominate. Increased prevalence of FH due to the founder effect can be detected in such populations as Ashkenazi Jews, Afrikaans-speaking white South Africans, French Canadians, Lebanese Christian Arabs, Icelanders and Finns (15).

2.3 Diagnostic of FH

Three groups have developed diagnostic tools for FH: The US MedPed Program, the Simon Broome Register Group in the United Kingdom, and the Dutch Lipid Clinic Network (18).

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2.3.1 Biochemical diagnostic

The MedPed criteria use cutpoints for total cholesterol levels specific to an individual's age and family history. Cholesterol levels vary with age, and in men and women, and they are population specific. The cut-off level for diagnosis of hypercholesterolaemia should thus ideally be gender, age and population specific. In FH patients HDL-levels are within the normal range (or low), and so it is more specific to make the diagnosis using LDLcholesterol levels. LDL-cholesterol is usually calculated using the Friedewald formula, and consequently a fasting lipid profile is required, which is less convenient and more costly, and so the diagnosis is usually made using total cholesterol levels in the first blood measurement (often non-fasting), and then LDL-cholesterol measurements for a second (fasting) test. However, cholesterol levels alone are not sufficient to confirm a diagnosis of FH because the range of blood cholesterol levels in FH overlaps with that of people with non-genetic polygenic hypercholesterolaemia, leading to a false positive and false negative rate of between 8 and 18% (19).

2.3.2 Clinical diagnostic

A diagnostic definition of FH, which supplements cholesterol measurements with clinical signs and family history has become widely used (Table 3.). The Simon Broome Register criteria in the UK, take into account that total and LDL levels differ for adults and children.

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Table 3 Simon Broome Familial Hypercholesterolemia Register diagnostic criteria for familial hypercholesterolemia

Criteria	Description
A	Total cholesterol concentration above 7.5 mmol/liter (290 mg/dl) in adults or a total cholesterol concentration above 6.7 mmol/liter(260 mg/dl) in children aged less than 16 years, or
	Low density lipoprotein cholesterol concentration above 4.9 mmol/liter(190 mg/dl) in adults or above 4.0 mmol/liter(150 mg/dl) in children
B	Tendinous xanthomata in the patient or a first-degree relative
C	DNA-based evidence of mutation in the LDLR or APOB gene
D	Family history of myocardial infarction before age 50 years in a second-degree relative or before age 60 years in a first-degree relative
E	Family history of raised total cholesterol concentration above 7.5 mmol/liter(290 mg/dl) in a first- or second-degree relative
Diagnosis	
A "definite" FH† diagnosis requires either criteria a and b or criteria c	
A "probable" FH diagnosis requires either criteria a and d or criteria a and e	

The criteria also take account of evidence of dominant transmission and the age of onset of coronary disease in the kindred. Using this approach, cases are categorised as ‘definite’ and ‘probable’. The presence of tendon xanthomata is the key feature in the ‘definite’ diagnosis of FH according to the criteria of the Simon Broome Register. These are most commonly found in the knuckles and in the Achilles tendons, and they are a result of cholesterol accumulation. Tendon xanthoma are usually not present until the fourth decade of life, and are therefore not helpful in systematic case finding among younger relatives. A family history of hyperlipidaemia or of premature coronary heart disease does not confirm a genetic cause or a monogenic pattern of inheritance. The specificity of family history of CHD

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as indicating a genetic risk depends strongly on the prevalence of coronary heart disease in the population being studied. A similar diagnostic tool has been developed by the Dutch Lipid Clinic Network. This includes similar features to the Simon Broome criteria, but adds the calculation of a numeric score (19).

2.3.3 Genetic diagnostic

A definitive diagnosis of FH may be made using DNA-based mutation screening methods. At present these methods are relatively expensive. However, once the causative mutation in the patient has been found, relatively cheap molecular testing in relatives is possible. Molecular testing is relatively easy in countries with only one or a few different mutations causing FH, but in the majority of countries with high genetic heterogeneity at the present time the mutation screening methods available fail to identify a mutation in all patients with a clinical diagnosis of FH, with detection rates in general being 30–50%. In the UK up to 5% of FH patients are reported to have large deletions of the LDLR gene, and deletions in the gene have been widely reported, and these are routinely detected with time-consuming Southern Blot methods, although PCR based methods have been reported for deletion screening. In a study using Single Strand Conformation Polymorphism (SSCP) screening, Southern blotting, DNA sequencing and RNA analysis, only 66% of patients had detected mutations. SSCP analysis is routinely reported to have a sensitivity of 75–85%, suggesting that a mutation may have been missed for technical reasons in possibly 15–25% of patients (15). Although the low detection rate in genetic testing may be due in part to the insensitivity of the methods or because not all of the LDLR and APOB genes are screened, it is probably also the result of misdiagnosis using the clinical and blood cholesterol criteria. Finally, in some patients mutations may not be present in the LDLR or APOB but occur in the unidentified FH3 gene on chromosome 1, and to date the relative contribution of mutations at this locus to FH is unknown (20,21).

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2.4 Current treatment method

2.4.1 Dietary intervention

Diet alone is not sufficiently effective for the treatment of heterozygous or homozygous FH. However, it should be the starting point of any management program. Dietary intervention relies upon the reduction of LDL-C by changing the type of fatty acids, reducing dietary cholesterol, including foods rich in soluble fiber that bind bile acids, and weight loss when indicated. Reducing cholesterol absorption with foods containing plant stanol- and sterol-esters may also prove effective. Both LDL-C production and removal rates appear to be affected by these dietary changes. Estimates suggest that a diet consisting of whole grains, legumes, fruits, vegetables, nuts, fishs, and fat-free dairy products, and no more than 100 mg/day of cholesterol, 20% of calories from fat and 6% from saturated fatty acids reduces plasma LDL-C levels by between 18 and 21% in heterozygous FH adults and children. LDL-C reductions of 30% from an initial mean of 260 mg/dl were seen in FH subjects changing from a diet rich in butter (34% fat calories) to diets rich in either salmon or safflower oil. Thus, FH patients, despite their genetically elevated LDL-C, are as responsive to diet as other hypercholesterolemic subjects (22).

2.4.2 Pharmacotherapy

The primary aim of drug therapy is to decrease plasma LDL-C. This may be achieved by increasing LDL removal via an increase in LDL receptors on the surface of hepatocytes and/or a decrease in LDL production. Treatment strategies that deplete liver cholesterol stores will upregulate LDL receptor gene transcription via the sterol-regulatory element-binding protein (SREBP) pathway. The bile sequestrants were the first class of drugs to exploit this pathway by preventing the absorption of bile acids from the ileum. Increased excretion of bile acids produces an increase in the conversion of cholesterol into bile salts, a decrease in liver cholesterol and a compensatory rise in LDL receptor synthesis by the liver. Although bile acid sequestrants have been shown to reduce plasma LDL-C levels by between 15 and 30% in heterozygous FH individuals, their effectiveness is limited by a simultaneous increase in de novo cholesterol synthesis which is also mediated by SREBP. Statins provide the most dramatic reductions in plasma LDL-C levels in heterozygous FH

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adults and children when compared with other lipid-reducing therapies. The statins act by blocking the HMG-CoA reductase enzyme, which catalyses the rate limiting step in de novo cholesterol synthesis. Competitive inhibition of this enzyme by the statins decreases hepatocyte cholesterol synthesis, which causes the heptocytes to synthesize more HMG-CoA reductase and LDL receptors. Although the increase in HMG-CoA reductase ensuring a steady state is maintained between cholesterol synthesis and utilization, the level of plasma LDL-C is reduced due to the increase in LDL-C receptors. There is clear evidence that statins also reduce the production of very low-density lipoprotein (VLDL) in the liver, and this may add to their LDL-C and triglyceride lowering effects (22).

2.4.3 Other treatment options

Patients with homozygous FH respond poorly to treatments known to be effective in heterozygotes because they produce few functional LDL receptors. Current treatment options for these individuals and nonresponsive heterozygous FH patients include LDL apheresis and liver transplantation. LDL apheresis uses a continuous-flow blood cell-separator to directly remove LDL from plasma. Data suggest that repeating this procedure every 1–2 weeks, in combination with nicotinic acid therapy, produces a reduction in the mean plasma cholesterol level of around 50%. Liver transplantation can successfully reduce plasma LDL-C levels by providing an external source of LDL receptors. However, this procedure is associated with substantial risks and recipients require life-long treatment with immunosuppressive drugs (22, 23).

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3. Low density lipoprotein receptor (LDLR)

3.1 LDLR protein and domain structure

The LDL receptor is a cell surface glycoprotein that contains approximately two asparagines-linked (N-linked) oligosaccharide chains of the complex type and approximately 18 serine/threonine-linked (O-linked) oligosaccharide chains. About two-thirds of the O-linked sugars are clustered in one region of the molecule (24). The receptor is first synthesized in the rough ER and then sent to the Golgi apparatus. The Golgi apparatus subsequently targets the LDLR to the cell surface, where they gather in clathrin-coated pits. Clathrin coated pits are specialized regions of the plasma membrane that are lined on the cytoplasmic surface by a protein called clathrin. After the LDLR binds to LDL, the coated pits internalize and the clathrin coat disintegrates. Multiple endocytic vesicles fuse to form endosomes where the pH begins to fall below 6.5. At this acidity, the LDL dissociates from the receptor while the LDLR returns to the cell surface. LDL that is dissociated from the receptor is delivered to the lysosome where hydrolytic enzymes degrade LDL for metabolic use (25).

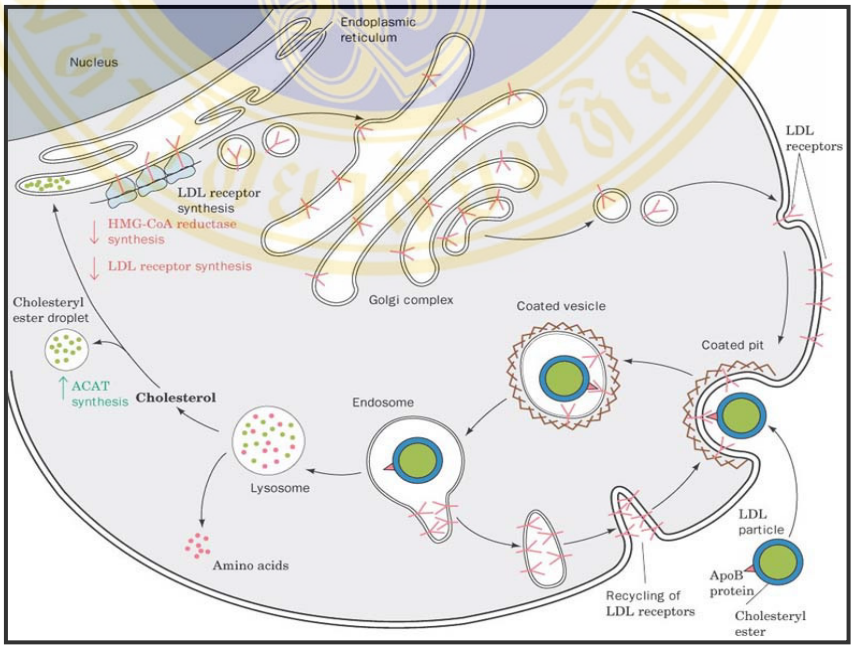

Figure 4 Cellular pathway of the LDLR
(http://www.med.unibs.it/~marchesi/biotec/slides/lipoproteine/LDL-receptors.jpg)

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The LDLR protein consists of five distinguishable domains (Fig.5). The first domain of the LDL receptor consists of the NH_2-terminal 292 amino acids, which is composed of a sequence of 40 amino acids that is repeated with some variation seven times. Each of the seven 40-amino acid repeats contains six cysteine residues. A striking feature of each cysteine-rich repeat sequence is a cluster of negatively-charged amino acids near the COOH-terminus of each repeat. The charges on these sequences are complementary to a cluster of positively-charged residues that are believed to occupy one face of a single α-helix in apo E, the best studied ligand for the LDL receptor. The second domain of the LDL receptor, consisting of - 400 amino acids, is 35% homologous to a portion of the extracellular domain of the precursor for epidermal growth factor (EGF). The third domain of the LDL receptor lies immediately external to the membrane-spanning domain and consists of a stretch of 58 amino acids that contains 18 serine or threonine residues. This domain is encoded within a single exon. Proteolysis studies reveal that this region contains the clustered O-linked sugar chains. The fourth domain consists of a stretch of 22 hydrophobic amino acids that is believed to anchor the receptor in the cell surface by spanning the membrane. The fifth domain is the cytoplasmic tail. It contain a COOH-terminal segment of 50 amino acids that projects into the cytoplasm. The cytoplasmic domain of the LDL receptor plays an important role in clustering in coated pits, either through interaction with clathrin itself or with some protein that is associated with clathrin on the cytoplasmic side of the membrane (24, 26).

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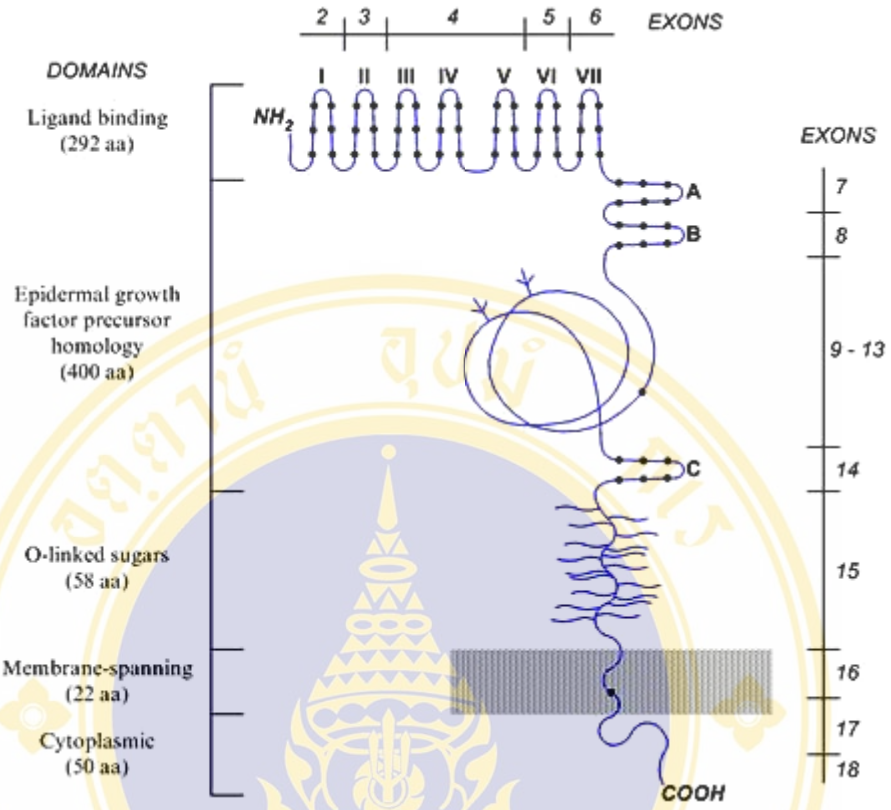

Figure 5 LDLR domains
(http://www.iemrams.spb.ru:8100/english/molgen/fh-en/domain-e.htm)

3.2 LDLR gene

The LDL receptor gene is located on the short arm of chromosome 19. It consists of 18 exons and 17 introns that span 45 kilobases (kb) (Fig.6). The gene encodes a single-chain transmembrane polypeptide of 839 amino acids, which consists of five functional domains. The amino-terminus of the protein contains the LDL-binding elements the recognize apolipoprotein B (apo B), the major apolipoprotein in LDL. The correspondence between gene exons and functional domains of the mature protein is well established. Exon 1 encodes the 21 amino acids of the signal sequence which is cleaved from the protein during translocation into the endoplasmic reticulum (ER). Exons 2–*6* encode the ligand-binding domain, which is made up of seven repeats of 40 amino acids each. Exons 7–14 encode a 400 amino acids sequence that is 33% identical to a portion of the human epidermal growth factor (EGF) precursor gene. Exon 15 encodes 58 amino acids that are enriched in serine and threonine

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residues, which serve as attachment sites for O-linked sugar chains. The 3' end of exon 16 and the 5' end of exon 17 encode the 22 hydrophobic amino acids of the membrane-spaning domain. The remainder of exon 17 and the 5' end of exon 18 encode the 50 amino acids that make up the cytoplasmic domain. The remainder of exon 18 specifies the 2.6 kb 3' untranslated region of the mRNA (27).

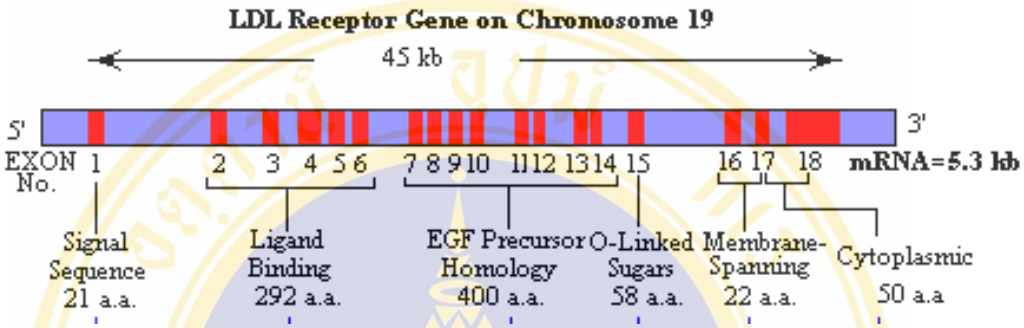

Figure 6 LDLR gene
(http://www.people.virginia.edu/~rjh9u/ldlrecep.html)

3.3 Mutations of LDLR gene

In general, mutations causing a premature stop codon, or a frame-shift mutation or a large deletion or re-arrangement of the gene, are all likely to result in the generation of a truncated protein which in almost all cases will be dysfunctional. If such a mutation is detected, it is almost undoubtedly the cause of FH in this patient. For missense mutations two types must be distinguished. The first are those that alter a critical amino acid, for example one that changes or adds a residue such as cysteine, or substitutes a large bulky amino acid such as Tryptophan for a small one such as Glycine (19). Mutations causing such substitutions are very likely to result in a defective LDL-receptor and therefore to be the cause of FH in the patient (19). The second group are those missense mutations that cause a conservative amino acid change, or that occur in a non-critical region of the protein, and these may not be FH-causing and their effect must be interpreted with caution (19). The analysis of the updated data gives the following informations: (i) 63% of the mutations are missense, and only 20% occur in CpG dinucleotides; (ii) although the mutations are widely distributed throughout the gene, there is an excess of mutations in exons 4 and 9, and a deficit in exons 13 and 15; (iii) the analysis of the distribution of mutations located

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within the ligand-binding domain shows that 74% of the mutations in this domain affect a conserved amino-acid, and that they are mostly confined in the C-terminal region of the repeats. Conversely, the same analysis in the EGF-like domain shows that 64% of the mutations in this domain affect a non-conserved amino-acid, and, that they are mostly confined in the N-terminal half of the repeats. The database is now accessible on the World Wide Web at http://www.umd.necker.fr (28, 29).

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Mutations in the LDLR gene have been classified into five functional groups based on the characteristics of the LDLR produced (Table 4.) (22,27).

Table 4 Classification of LDL receptor gene mutations

Class of mutation	Nature of the mutation
1	Gene fails to produce immunoprecipitable protein (null alleles).Variety of mutations including promoter deletions, splice site mutations, frameshift mutations and other large deletions. Some nonsense mutations may cause accelerated receptor protein degradation.
2	Proteins are encoded that are blocked, either completely (2a) or partially (2b), in transport between the endoplasmic reticulum and Golgi complex (transportdefective alleles). Abnormal folding of the protein appears to be the basis of the blocked transport. Most defects are in the binding and EGF precursor homology domains.
3	Gene encodes proteins that are synthesized and transported to the cell surface normally, but fail to bind LDL (binding-defective alleles). Defects are in the binding and EGF precursor homology domains.
4	Proteins are encoded that move to the cell surface and bind LDL normally, but are unable to cluster in clathrin-coated pits and thus do not internalize LDL (internalization-defective alleles). Defects are in the Cytoplasmic domain.
5	Gene encodes receptors that bind and internalize ligand in coated pits, but fail to discharge the ligand in the endosome and do not recycle to the cell surface (recycling-defective alleles). Defects are in the EGF precursor homology domain.

4. Mutation detection methods

Analysis of DNA variation (polymorphisms and mutations) is one of the most common challenges faced by molecular biologists. Studies of polymorphisms and mutations as molecular markers of or underlying causes of disease have confirmed the importance of mutations and polymorphisms detection. With mutation detection currently being so important for the study of genetic diseases, gene discovery, and

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solving problems of basic biology, there is a large demand for quick and relatively cheap methods for mutation detection. Therefore, many different methods have been developed for detecting new mutations and screening populations for known mutations or polymorphisms (29). Mutation detection can be performed in two fundamental processes.

-To detect mutations for the first time in a piece of DNA: This is a search and is referred to as screening for, or discovery of, mutations.

- To detect on subsequent occasions mutations in DNA once they have been detected initially. This may be for diagnosis of disease in a family, when the methods are referred to as diagnostic methods, or in the case of population studies when assessing single nucleotide polymorphism for linkage or other studies, when the methods are called genotyping methods (30).

4.1 Single-Strand conformation polymorphism (SSCP)

Single-strand conformational polymorphism (SSCP) is one of the most widely used mutation scanning techniques because of its simplicity. The principle of this method is based on the fact that the electrophoretic mobility of nucleic acids in a non-denaturing gel is sensitive to both size and shape. Unlike double-stranded DNA, single-stranded DNA is flexible and will adopt a conformation determined by intramolecular interactions and base stacking that is uniquely dependent on sequence composition (Orita *et al.*, 1989). This conformation can be affected when even a single base is changed. Conformational changes can be detected as alterations in the electrophoretic mobility of the single-stranded DNA in non-denaturing polyacrylamide gels. SSCP is still largely empirical because of the absence of a robust theory for predicting the dependence of conformation on sequence, and of mobility on conformation. SSCP is, nonetheless, a convenient mutation screening method. Detection levels of 70% to greater than 95% in certain model systems have been achieved. Achieving such high detection rates by SSCP may require running gels under several conditions, because the conformation of single-stranded DNA is sensitive to a number of parameters, including ionic strength, the type of gel matrix,

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fragment length, and temperature. Originally, SSCP was performed on large sequencing gels in a cold room to control temperature and using radioactive nucleotides. The procedure was cumbersome and not always reproducible. The developments of non-radioactive techniques and temperature-controlled electrophoretic units, and the modification of the technique for standard and mini-gels, have increased the simplicity and reproducibility of this method. Basic Protocol: The target sequence from genomic DNA or cDNA is amplified by PCR. The amplicon is denatured to single-stranded DNA and electrophoresed on a non-denaturing gel. Bands of the single-stranded DNA at positions in the gel different from the wild-type indicate the presence of a mutation. In general, a wild-type sample will generate two SSCP bands. Similarly, a homozygous mutant sample will also generate two SSCP bands, but these will migrate differently from the wild-type. If it is a heterozygous mutant, four bands will be generated: two with the same mobility as the wild-type and two with different mobility. There may be only three bands if the mutation changes the conformation of one strand but not the other. Generating the product DNA products used for SSCP is generally amplified by PCR. The sensitivity of PCR-SSCP tends to decrease as fragment length increases. For fragments of about 200 bp, greater than 90% of single-base changes can be detected; whereas for fragments of 400 bp, the detection rate is lowered to 80%. Although mutations have been detected in fragments as large as 800 bp, the sensitivity of the assay will probably not be as high. Sequences longer than 400 bp should be divided into shorter segments before analysis by SSCP. This can be done by generating over-lapping subfragments separately or by amplifying the intact fragment and then digesting it with restriction enzymes. It is important that PCR conditions be optimized for stringency, as artifacts will interfere with the assay. The PCR product should be checked prior to SSCP analysis (38).

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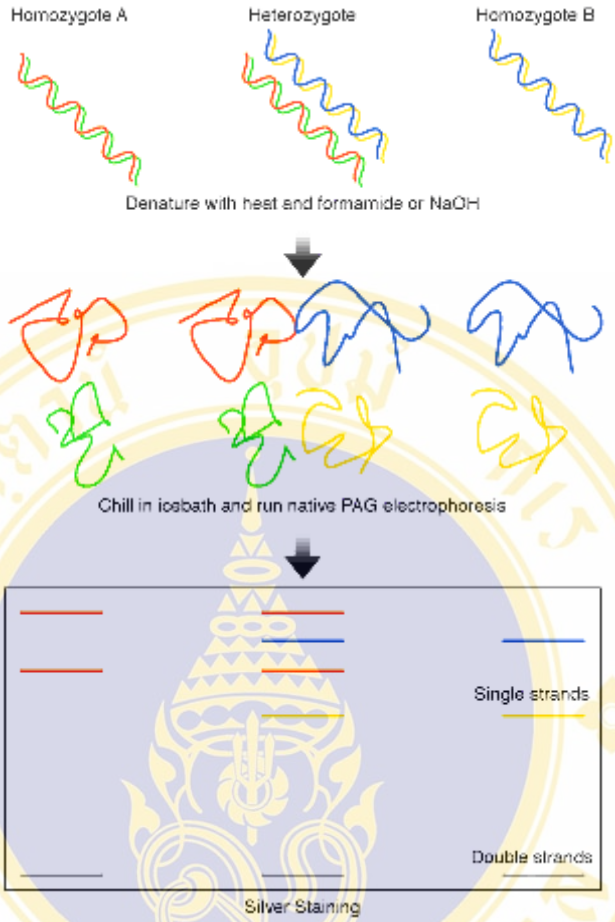

Figure 7 Mutation detection with SSCP (38)

Takao Maruyama *et al.* (1995) developed simple SSCP methods for detecting these mutations and investigated four Japanese FH homozygotes and identified five point mutations: a splice site mutation in intron 12 (the 1845+2 T→C mutation), a missense mutation in exon 7 (the C317S mutation), a nonsense mutation in exon 17 (the K790X mutation), a missense mutation in exon 14 (the P664L mutation), and a missense mutation in exon 4 (the E119K mutation)(32). Humphries *et al.* (1997) developed SSCP for high-throughput mutational analysis and applied screen for mutations in the LDL receptor gene in patients with familial hypercholesterolemia (33). Using SSCP, Lee *et al.* (1998) also detected mutations in exon 4 of the LDLR gene in 15 of 80 of these individuals; 7 of 15 had the same C163Y mutation (34). Jensen *et al.* (1996) performed the PCR-SSCP analysis. This method was as sensitive and efficient as DNA sequencing in the ability to identify the sequence variations in the LDLR gene of patients with heterozygous and in 2002, they have shown that the

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PCR-SSCP analysis is a highly efficient and sensitive technique for detection of mutations in the 18 exons including intronic splice-site sequences and the promoter region of the LDL receptor gene in FH patient in Denmark (35, 36).

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CHAPTER III
MATERIALS AND METHODS

MATERIALS

1. Subjects

DNA samples from a group of unrelated patients with primary hypercholesterolemia from Departmaent of Preventive Medicine, Siriraj Hospital (N=11) and Navy hospital (N=13) were invited to participate in this study. Diagnosis of primary hypercholesterolemia (hyperlipidemia type IIa) was based on the presence of increased plasma total cholesterol and LDL-cholesterol before lipid-lowering therapy: total cholesterol levels ≥ 290 mg/dl, LDL-cholesterol levels ≥ 190 mg/dl and triglyceride levels ≤ 200 mg/dl. Patients with secondary causes of hyperlipidemia such as diabetes, hypothyroidism or hyperthyroidism and nephrotic syndrome were excluded. The normalipidemic subjects (N=100) with total cholesterol levels ≤ 200 mg/dl, LDL-cholesterol levels ≤ 130 mg/dl and triglyceride levels ≤ 200 mg/dl were recruited as control. Informed, written consents were obtained from all participants.
The study was approved by the Committee on Human Rights Related to Human Experimentation Mahidol University and the Ethical Committee on Research Involving Human Subject, Faculty of Medicine Siriraj Hospital, Mahidol University.

2. Oligonucleotide Primers

The whole coding region of the LDL receptor gene (promoter and 18 exons) were amplified by PCR. For the amplification reactions, we used oligonucleotide primers designed by Charlotte *et al.* (promoter), Pocsai *et al.* (exon 4), Leitersdorf *et al.* (exon 1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 17) and Hobbs *et al.* (exons 15 and 18). The primer sequences, size of amplicons, and respective annealing temperatures were listed in Table 5.

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Table 5 Sequences of oligonucleotides used in PCR assay for amplifying all exons and the promoter region of the LDLR gene.

Region/ Exon	Primer Name	Oligonucleotide sequence (Sense & antisense, 5' to 3')	Size (bp)	T_a (°C)
Promoter	Forward	GAG GCA GAG AGG ACA ATG GC	380	62
	Reverse	CCA CGT CAT TTA CAG CAT TTC AAT G		
Exon 1	SP90	CAT TGA AAT GCT GTA AAT GAC GTG G	210	58
	SP91	TTC TGG CGC CTG GAG CAA GCC TTA C		
Exon 2	SP57	CCT TTC TCC TTT TCC TCT CTC TCA G	183	58
	SP58	AAA ATA AAT GCA TAT CAT GCC CAA A		
Exon 3	SP59A	TGA CAG TTC AAT CCT GTC TCT TCT G	178	58
	SP60B	AAT AGC AAA GGC AGG GCC ACA CT		
Exon 4	P4.1	TGC AGC CCC CAA GAC GTG CT	386	58
	P4.2	CGC AGT TTT CCT CGT CAG AT		
Exon 5	SP62	CAA CAC ACT CTG TCC TGT TTT CCA G	173	68
	SP63	GGA AAA CCA GAT GGC CAG CGC TCA C		
Exon 6	SP64	TCC TTC CTC TCT CTG GCT CTC ACA G	173	58
	SP65	GCA AGC CGC CTG CAC CGA GAC TCA C		
Exon 7	SP66	AGT CTG CAT CCC TGG CCC TGC GCA G	169	68
	SP67	AGG GCT CAG TCC ACC GGG GAA TCA C		
Exon 8	SP68	CCA AGC CTC TTT CTC TCT CTT CCA	176	62
	SP69	CCA CCC GCC GCC TTC CCG TGC		
Exon 9	SP70	CCT GAC CTC GCT CCC CGG ACC CCC A	223	70
	SP71	GGC TGC AGG CAG GGG CGA CGC TCA C		
Exon 10	SP72	ATG CCC TTC TCT CCT CCT GCC TCA G	278	68
	SP73	AGC CCT CAG CGT CGT GGA TAC GCA C		
Exon 11	SP74	CAG CTA TTC TCT GTC CTC CCA CCA G	173	68
	SP75	TGG CTG GGA CGG CTG TCC TGC GAA C		
Exon 12	SP76	TCT CCT TAT CCA CTT GTG TGT CTA G	188	58
	SP77	CTT CGA TCT CGT ACG TAA GCC ACA C		
Exon 13	SP78	GTC ATC TTC CTT GCT GCC TGT TTA G	219	54
	SP79	GTT TCC ACA AGG AGG TTT CAA GGT T		
Exon 14	SP80	CCT GAC TCC GCT TCT TCT GCC CCA G	204	60
	SP81	ACG CAG AAA CAA GGC GTG TGC CAC A		
Exon 15	FH16	GAA GGG CCT GCA GGC ACG TGG CAC T	246	68
	FH35	GTG TGG TGG CGG GCC CAG TCT TTA C		

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Table 5 Sequences of oligonucleotides used in PCR assay for amplifying all exons and the promoter region of the LDLR gene. (Continued)

Exon 16	SP84 SP85	CCT CAC TCT TGC TTC TCT CCT GCA G CGC TGG GGG ACC GGC CCG CGC TTA C	128	62
Exon 17	SP86 SP87	TGA CAG AGC GTG CCT CTC CCT ACA G TGG CTT TCT AGA GAG GGT CAC ACT C	207	58
Exon 18	FH19 FH38	TCC GCT GTT TAC CAT TTG TTG GCA G AAT AAA ACA AGG CCG GCG AGG TCT C	135	68

3. Enzymes

All of the enzymes used in this study were molecular biology grade. HaeIII and HpaII restriction endonucleases were purchased from BioLabs, USA. *Taq* DNA polymerase used for PCR and PCR-multiplex SSCP was expressed from a recombinant plasmid according to the Protocol of Pluthero (39). Commercial *Taq* polymerase perchased from Fermentus (Germany) was used to confirm the results and to perform DNA sequencing.

4. Chemical substances

Table 6 List of chemical substances

Chemical substances	**Molecular Weight**	**Source**
Acrylamide (C_3H_5NO)	71.08	BDH, England
Absolute ethanol (C_2H_2OH)	46.07	Merck, Germany
Agar-C	-	Bio Basic Inc., Canada
Agarose	-	SeaKem, USA
Ammonium acetate	-	Sigma, USA
Ammonium chloride	53.49	Sigma, USA
Ammonium Persulfate ($(NH_4)_2S_2O_8$)	228.20	Sigma, USA
Ampicillin ($C_{16}H_{18}N_3ONaO_4S$)	371.4	Bio Basic Inc., Canada
Boric Acid (H_3BO_3)	61.83	Merck, Germany
Bromophenol Blue($C_{19}H_9Br_4O_5SNa$)	691.9	USB, Austria
Bromophenol Blue-Xylene Cyanole Dye solution		Sigma, USA
Calcium Chloride	147	Sigma, USA
Deionized Formamide	-	Bio Basic Inc, Canada

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Table 6 List of chemical substances (Continued)

Chemical substances	Molecular Weight	Source
2'-Deoxynucleotide 5' triphosphate (dNTP)	-	BioBasic Inc, Canada
DL-Dithiothreitol (DTT)($C_4H_{10}O_2S_2$)	154.25	BioBasic Inc, Canada
DNA Ladder Marker	-	Fermentus, Germany
Dimethyl Sulfoxide (DMSO) $(CH_3)_2SO$	78.13	Merck, Germany
Ethidium bromide ($C_{21}H_{20}N_3Br$)	394.31	Sigma, USA
Ethylenediamine tetraacetic acid (EDTA) (disodium salt, dehydrate)	372.24	Bio Basic Inc., Canada
37% Formaldehyde (CH_2O)	30.0	Merck, Germany
Formamide dye mix	-	Amresco, USA
Glacial Acetic Acid (CH_3COOH)	60.05	Merck, Germany
Glycerol ($HOCH_2CH$ (OH) CH2OH)	92.09	Merck, Germany
Guanidine-HCl $(NH_2)_2$-C=NH.HCl)	95.53	Sigma, USA
Hydrochloric	36.46	Merck, Germany
IGEPAL	-	Sigma, USA
Isopropyl-β-D-Thiogalactopyranoside (IPTG)	-	Sigma, USA
($C_9H_{18}O_5S$)	238.31	Sigma, USA
Lysozyme	-	BioBasic,Inc, Canada
Magnesium Sulfate ($MgSo_4 .7H_2O$)	246.5	Sigma, USA
Magnesium Chloride ($MgCl_2.6H_2O$)	203.30	Sigma, USA
N, N'-methylene-bis acrylamide ($C_7H_{10}N_2O_2$)	154.2	Sigma, USA
N, N,N' N'-Tetramethyl-ethylenediamine (TEMED)	116.2	Bio Basic Inc., Canada
PCR purification Kit	-	QIAGEN, Germany
Potassium chloride	74.5	Sigma, USA
Proteinase K	-	Invitrogen, Germany
Silver Nitrate ($AgNO_3$)	169.87	Merck, Germany
Sodium carbonate ($Na_2 CO_3$)	105.99	Merck, Germany
Sodium chloride	58.44	Bio Basic Inc., Canada
Sodium dodecyl sulphate (SDS, $C_{12}H_{25}O_4SNa$)	288.4	Sigma, USA
Sodium hydroxide (NaOH)	40.0	Merck, Germany
Tris (hydroxymethyl)aminomethane, ($C_4H_{11}NO_3$)	121.4	Research Organics,USA
Tryptone powder	-	Bio Basic Inc., Canada

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Table 6 List of chemical substances (Continued)

Chemical substances	Molecular Weight	Source
Tween 20	-	Research Organics, USA
Xylene cyanol FF	554.6	Research Organics, USA
Yeast Extract	-	BioBasic Inc., Canada

5. Instruments

Table 7 List of instruments

Instruments	Source
Autoclave	Hirayana, Japan
Centrifuge	Heraeus Sepatech, USA
DNA Thermal cycler (Gene Amp PCR system 2400, 9700)	Perkin Elmer, USA
Horizontal agarose gel electrophoresis set	Mupid II, Japan
Hoefer SE260	Pharmacia Biotech, USA
Hotplate stirrer SM22	Stuart Scientific, UK
Hot air oven	Memmert, Germany
Incubator	Heraeus, Germany
Microcentrifuge	Hettich, Germany
pH meter	Orion Research, USA
Pipetteman	Gilson, France
Poraloid Camera (Fotodyne)	-
Power supply	Pharmacia Biotech, USA
Refrigerator	Sharp, Japan
Spectrophotometer	Shimudzu, Japan
Spin down	-
UV-Transilluminator	Herolab, Germany
Vortex mixer	IKA Work Inc., USA
Vertical electrophoresis set	Thailand
Water bath	Precision Qualitron Inc., USA

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6. Miscellaneous

Table 8 List of miscellaneous

Miscellaneous	**Source**
Cellophan membrane	Thailand
Camera film (Polaroid)	USA
Glove	Thailand
Microcentrifuge tube (0.6, 1.5ml)	Scientific Plastic, USA
Parafilm	Chicaco,USA
Plastic centrifugue tube (15, 50 ml)	Elkay, UK
Plastic trays	Thailand
Thin wall PCR reaction tube (0.2 ml)	Scientific Plastic, USA
Micropipette tips (0.2, 200, 1000 μl)	Sorenson, Promega, USA
Nalgene filter	Pall Corporation, USA
Whatman Filter paper	Whatman Ltd, England

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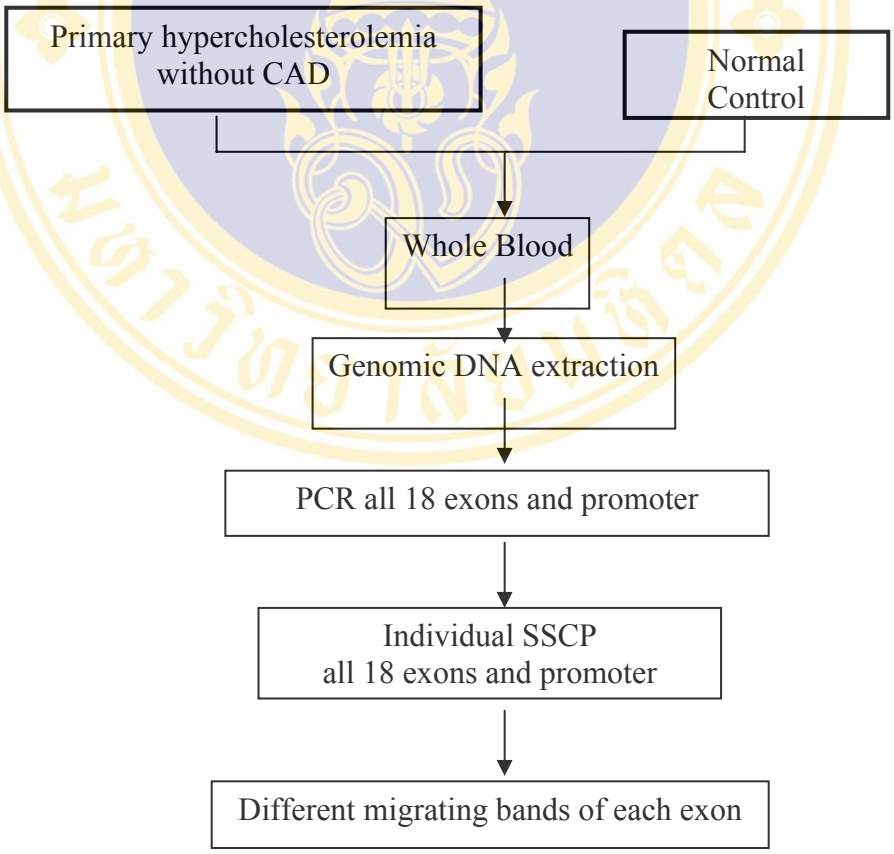
METHODS

1. Determination of lipid profiles

Lipid profiles were analyzed by Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University. Venous blood (5-10 ml) was taken after 12-14 hours of fasting. Plasma cholesterol and triglyceride levels were determined with automation by Hitachi 917 Autoanalyzer. The concentration of plasma HDL was measured after precipitation of LDL and VLDL fractions with dextran sulfate and $MgCl_2$ and plasma LDL level was calculated using the formula described by Friedewald *et al* (40).

The experimental design presented in Fig.8. The methods used in this study are described as followings.



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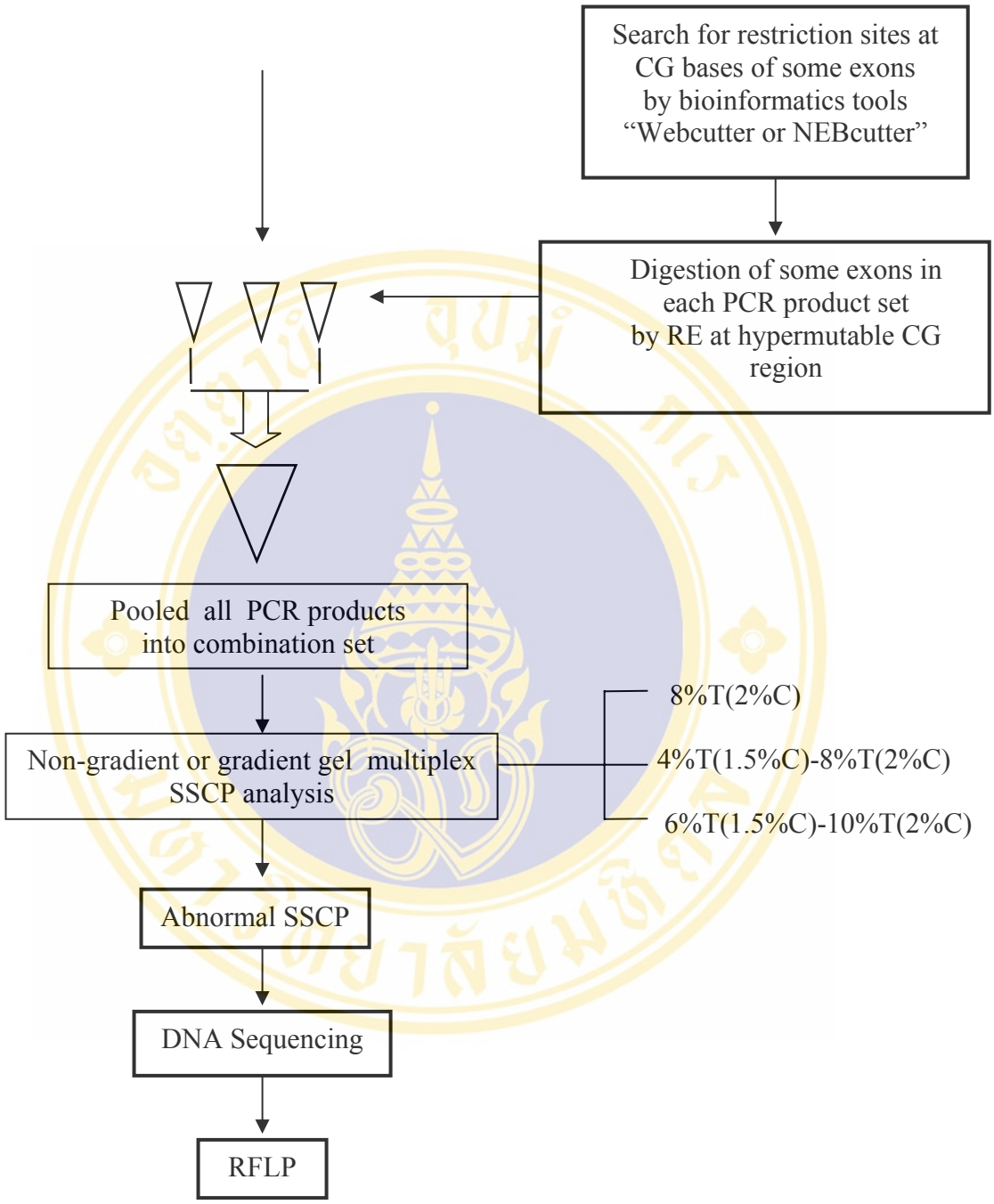

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2. Genomic DNA extraction

2.1 Reagent preparations

2.1.1 10 N NaOH

The preparation of 10 N NaOH involved a highly exothermic reaction which can cause breakage of glass containers. This solution was prepared with extreme care in plastic breakers. To 200 ml of sterile water for injection slowly add 100 g of NaOH pellets was slowly added with continuous stirring in a breaker placed on ice. When the pellets have dissolved completely, the volume was adjusted to 250 ml with sterile deionized water. The solution was stored in a plastic container at room temperature. Sterilization was not necessary.

2.1.2 5%EDTA, pH 7.4

25 g of EDTA disodium salt was weighed and dissolved in 400 ml of sterile deionized water. The pH of the solution was adjusted to 7.4 with 10 N NaOH. The volume of the solution was adjusted to 500 ml. The solution was sterilized by autoclaving.

2.1.3 Solution A, pH 7.2

6.35 g of NH_4Cl, 1.33 g of EDTA and 0.92 g of Trizma base were weighed and dissolved with 800 ml of distilled water. The pH of the solution was adjusted to 7.2 with HCl and the volume was adjusted to 1 liter. The solution was autoclaved. Working solution A was prepared by diluting solution A with 2 volume of sterile deionized water before use.

2.1.4 Proteinase K (10mg/ml)

A lyophilized powder of proteinase K was dissolved at a concentration of 10 mg/ml in sterile deionized water. The stock solution was into small aliquots and stored at -20°C until use.

2.1.5 10% SDS

10 g of SDS was weighed and dissolved with sterile deionized water. The solution was stirred until it was homogeneous and the volume was adjusted to 100 ml. The solution was incubated at 56 ^{0} C for 1-2 hours until it was dissolved. This solution was used with no need to be autoclaved.

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2.1.6 1 M Tris-HCl, pH 7.6

60.6 g of Tris-HCl was weighed and dissolved with sterile deionized water. Approximately 13 ml of 6 M HCl was added to the solution to adjust pH to 7.6. The volume of the solution was adjusted to 500 ml. The solution was sterilized by autoclaving.

2.1.7 7.5 M Guanidine-HCl, pH 7.6

72.0 g of Guanidine-HCl was weighed and approximately 10 ml of 1 M Tris-HCl was added to adjust the pH to 7.6. The volume of the solution was adjusted to 100 ml with autoclaved sterile deionized water. The solution was filtered by 0.2 μm Nalgene filter.

2.1.8 0.5 M EDTA, pH 8.0

93.0 g of EDTA disodium salt was weighed and approximately 400 ml of sterile water for injection and 10.0 g of NaOH pellets were added. The pH of the solution was adjusted to 8.0 with 10 N NaOH. The solution was sterilized by autoclaving.

2.1.9 TE 10-1 buffer

10 ml of 1 M Tris-HCl, pH 7.6 and 2 ml of 0.5 M EDTA, pH 8.0 were diluted in sterile deionized water and the volume were adjusted to 1 Liter. The solution was sterilized by autoclaving.

2.2 Procedure

Genomic DNA was isolated from peripheral leukocytes in 10 ml EDTA blood samples by Guanidine-HCl method described previously (UCLA,1993). Ten millilitres of whole blood were collected into 50 ml capped tube which contained 400 μl of 5%EDTA. Two volumes of working solution A was added to the whole blood, mixed by vortex the pellet to prevent clumping and left for 10 minutes at room temperature. The pellet was collected by centrifugation at 3,000 rpm for 10 minutes. The supernatant was discarded. Two volumes of working solution A was added to the pellet and the previous step was repeated until supernatant was clear. This step was not repeated more than three times. The supernatant was discarded and the packed WBC was added by the following reagents. First, 400 μl of sterile water and 40 μl of Proteinase K was added into the packed WBC and then mixed with 1ml pipette-tip in order to thoroughly mix the content. Second, 400 μl of autoclaved sterile water was

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added and resuspended further. Third, 300 µl of 10% SDS was added, and the sample was mixed gently by rocking the tube back and forth, air-bubble was avoided. After mixing, the solution was incubated at 37 ^{0}C for overnight. Finally, 300 µl of 7.5M Guanidine-HCl was added and gently mixed. The whole solution was mixed vigorously and incubated at 68-70 ^{0}C for 1 hour. The mixture was centrifuged at 3,000 rpm for 10 minutes at 4 ^{o} C. Only the above clear solution was gently mixed and reincubated at 68-70 ^{0}C for additional 30 minutes. The mixture was recentrifuged as above. The supernatant was transferred to the new labeled 50 ml cap tubes with 5 ml of cold absolute ethanol. The tube was gently rock back and forth until cotton- like strands of DNA was appearent and then the mixture was stored at –20 ^{0}C for 1 hour. The cotton-like strand of DNA was transferred with 800 ul of cold absolute ethanol to a new 1.5 ml eppendorf tube. The mixture was centrifuged at 10,000 rpm for 5 minutes and the alcohol supernatant was discarded. 800 µl of 70% ethanol was added to the sample. The sample was then inverted to loosen the pellet and the sample was let strand for 1 minute. The mixture was centrifuged at 10,000 rpm for 5 minutes and let stand the supernatant was discarded. The DNA sample was then subjected to air dry at 37 ^{0} C for 2 hours or at room temperature until the sample was dry with the cap open to evaporate the ethanol. 100-250 µl of TE 10-1 buffer was added to the sample and the sample was incubated at 37 ^{0}C for overnight. The sample was stored at –20 ^{0}C until use. To determine the integrity of DNA, 3 µl of DNA was mixed with 2 µl loading dye and analyzed on 1% agarose gel electrophoresis in 1X TBE buffer comparing with λDNA/HindIII fragment. The agarose gel was then stained with ethidium bromide and visualized by UV Transilluminator. The gDNA quality was verified by Polymerase Chain Reaction and agarose gel electrophoresis.

3. Polymerase Chain Reaction (PCR)

3.1 For multiplex PCR assay

3.1.1 Reagent preparation

3.1.1.1 10XPCR buffer (20mM $MgCl_2$)

80 µl of 25 mM $MgCl_2$ stock and 20 µl of 10X *Taq* Bufer with KCl were mixed in 600ml eppendorf tube.

3.1.1.2 10mM dNTPs

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10 µl of each 100 mMdNTPs (dATP,dGTP, dTTP,dCTP) were aliquoted and diluted to 100 µl with autoclaved sterile deionized water.

3.1.1.3 *Taq DNA polymerase*

As described in Appendix A.

3.1.2 Procedure

Each 25 µl reaction mixture consists of 0.1-0.3 µg of genomic DNA, 1X PCR buffer (2 mM $MgCl_2$), 2 mM each of dNTP, 1µM of each oligonucleotide primer and 1U Taq polymerase. After initial denaturation, 35 cycles were performed in a programmable DNA thermal cycler (ThermoHybaid PCR) using the temperature profile of 1 min at 94°C, 1 min at 49°C (annealing temperature) and 2 min at 72°C. The primer extension of the 35th cycle was extended to 12 min. The multiplex PCR products were electrophoresed on a 2% agarose gel in 1X TBE buffer for 30 min at a voltage of 100V. The fragments were dyed with ethidium bromide and were visualised by ultraviolet illumination. And the multiplex PCR products from DNA samples of primary hypercholesterolemia and control subject were subsequently used for SSCP analysis.

3.2 For multiplex SSCP assay

3.2.1 Reagent preparation

3.2.1.1 10XPCR buffer (15mM $MgCl_2$)

60 µl of 25 mM $MgCl_2$ stock and 40 µl of 10X *Taq* Bufer with $(NH_4)_2SO_4$ were mixed in 600ml eppendorf tube.

3.2.1.2 10XPCR buffer (20mM $MgCl_2$)

80 µl of 25 mM $MgCl_2$ stock and 20 µl of 10X *Taq* Bufer with $(NH_4)_2SO_4$ were mixed in 600ml eppendorf tube.

3.2.1.3 10mM dNTPs

10 µl of each 100 mMdNTPs (dATP,dGTP, dTTP,dCTP) were aliquoted and diluted to 100 µl with autoclaved sterile deionized water.

3.2.1.4 *Taq DNA polymerase*

As described in Appendix A.

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3.2.2 Procedure

Each 25 µl reaction mixture consists of 0.1-0.3 µg of gDNA, 1X PCR buffer (1.5-2mM $MgCl_2$), 0.2 mM each of dNTP, 0.4µM each of oligonucleotide primer and 0.5U *Taq* polymerase. After initial denaturation at 95°C 5 min, 35 cycles was performed in a programmable DNA thermal cycler (GeneAmp® PCR System 2400 or 9700) using the temperature profile of 1 min at 95°C, 1 min at annealing temperature (Table. 5) and 1 min at 72°C. The primer extension of the 35th cycle was extended to 5 min. The PCR products will be electrophoresed on a 2% agarose gel in 1X TBE buffer for 30 min at a voltage of 100V. The fragments will be dyed with ethidium bromide and visualised by ultraviolet illumination. PCR products from DNA samples of primary hypercholesterolemic and control subjects were subsequently combined into a set of multiplex for SSCP analyses. Each set was composed of 3 or 4 exons in 1:1:1 or 1:1:1:1 ratio, respectively. (For RE-digested amplified exon, 2 volumes were applied in the relevant combination set.)

4. Restriction digestion

Restriction endonucleases are enzymes that cleave DNA at specific sequences, yielding defined fragments of DNA molecules. They can be used to cleave a DNA molecule at unique site. In this study, there were two reasons to use this technique; first to generate the optimal sensitivity for SSCP of LDLR gene. Second, LDLR gene is a large gene which contains many hypermutable CG regions. RE digestion of hypermutable CG regions in the LDLR gene is expected to find new mutations and polymorphisms in this gene.

4.1 Procedure

Ten µl of PCR reactions were digested with the relevant restriction enzymes in the buffer recommended by the manufacturer. The reaction mixture will be incubated at 37°C for over night.

5. Electrophoresis

5.1 Agarose gel electrophoresis

5.1.1 Reagent preparation

5.1.1.1 10XTBE

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108 g of Trisma base, 55g of Boric acid and 9.3 g of EDTA disodium salt were weighed and dissolved with sterile deionized water. The volume of the solution was adjusted to 1 liter and mixed.

5.1.1.2 1X TBE buffer

100 ml of 10x TBE buffer was diluted to 1 litre with distilled water.

5.1.1.3 1X loading dye

0.125 g each of bromophenol blue and xylene cyanol FF were weighed and 15 ml of glycerol was added. The volume of the mixture was adjusted to 50 ml with autoclaved sterile water for injection.

5.1.1.4 100 bp DNA Ladder marker

5 µl (0.5µg) of stock Ladder Marker and 5 µl of 6X loading dye solution were taken and then 20 µl of steriled water was added.

5.1.1.5 1% Agarose gel electrophoresis

1 g of agarose was weighed and dissolved in 100 ml of 1X TBE buffer. The mixture was heated. The melting gel was poured into an electrophoresis chamber set with a comb inserted. The agarose gel was left to polymerize at room temperature for 30 min.

5.1.1.6 2% Agarose gel electrophoresis

2 g of agarose was weighed and dissolved in 100 ml of 1X TBE buffer. The mixture was heated. The melting gel was poured into an electrophoresis chamber set with a comb inserted. The agarose gel was left to polymerize at room temperature for 30 min.

5.1.1.7 Ethidium bromide solution (10 mg/ml)

1 g of ethidium bromide was weighed and dissolved in 100 ml of reverse osmosis water. The container was wrapped in aluminum foil or the solution was transferred to a dark bottle and store at room temperature.

5.1.2 Procedure

5.1.2.1 Analysis of PCR amplification

PCR products were checked for successful amplification on 2% (w/v) agarose gel in 1XTBE buffer. The gel was allowed to cool and solidify for 30 min. The gel was immersed in 1xTBE buffer at a depth of 1-2 mm. 5 µl of PCR

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amplified products were pipetted and mixed with 2 µl of gel loading buffer. The mixtures were loaded in each well on 2% agarose gel and electrophoresed for 20-30 minutes at 100 volts. After electrophoresis, the PCR products were stained with ethidium bromide solution for 1-2 minutes and visualized under ultraviolet light. The size of fragment was estimated by comparison with 100 bp DNA ladder marker under the same condition.

5.1.2.2 Analysis of PCR product purification

PCR products were purified on 1% agarose gel in 1X TBE. The gel was allowed to cool and solidify for 30 min. The gel was immersed in 1xTBE buffer at a depth of 1-2 mm. 100 µl of PCR amplified products were pipetted, mixed with 10 µl of gel loading buffer and 10 µl of ethidium bromide solution. The mixtures were loaded in each well on 1% agarose gel and electrophoresed for 20-30 minutes at 100 volts. After electrophoresis, the PCR products were visualized under ultraviolet light.

5.2 Acrylamide gel electrophoresis

5.2.1 Reagent preparation

5.2.1.1 50% (49:1) stock acrylamide solution

49 g of acrylamide and 1 g of N, N'-methylene-bis-acrylamide were weighed and dissolved in 100 ml sterile water for injection. The solution was filtered through Whatman paper no.1, kept away from light and stored at 4^{0}C.

5.2.1.2 20% ammonium persulfate

0.2 g of ammonium persulfate was weighed and dissolved in 1 ml of sterile water for injection. The solution was freshly prepared, kept away from light and stored at 4^{0}C.

5.2.2 Procedure

Polyacrylamide gel for determination of PCR products was prepared by dilution of the stock 50% acrylamide solution (49:1(w/v) of acrylamide and N, N'-methylene-bis-acrylamide) to give 8% polyacrylamide solution. For Hoefer SE260 apparatus : 1.6 milliliters of the 50% acrylamide stock solution and 0.5 ml of 10xTBE buffer were pipetted and adjusted to a total volume of 10 ml with sterile water for injection. After mixing, the solution was added with 50 µl of 20%

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ammonium persulfate and 5 µl of TEMED and mixed. The gel solution mixture was immediately poured between the minigel glass plates using 1000 µl pipette until the glass plate sandwich was full. A comb was placed in the assembled gel sandwich. The gel was left to polymerize for at least 30 minutes at room temperature. 2 µl of loading buffer and 5 µl of digested PCR products were mixed, and then the mixtures were loaded in each well of 8 % polyacrylamide gel. An electric current was applied at 100 volts for 60 minutes to resolved DNA fragments. After electrophoresis, the gel was stained by ethidium bromide solution for 1-2 minutes and visualized under ultraviolet light. The size of PCR fragment was estimated by comparison with 100 bp DNA ladder marker under the same condition.

6. Single strand conformation polymorphism (SSCP)

Single-strand conformational polymorphism (SSCP) is one of the most widely used mutation scanning techniques because of its simplicity. The principle of this method is based on the fact that the electrophoretic mobility of nucleic acids in a non-denaturing gel is sensitive to variation of both size and shape. Unlike double-stranded DNA, single-stranded DNA is flexible and will adopt a conformation determined by intramolecular interactions and base stacking that is uniquely dependent on sequence composition (Orita et al., 1989). This conformation can be affected when even a single base is changed. Conformational changes can be detected as alterations in the electrophoretic mobility of the single-stranded DNA in non-denaturing polyacrylamide gels.

6.1 Reagents preparation

6.1.1 50% (49.25:0.75) acrylamide stock solution with 1.5% cross link

49.25 g of acrylamide and 0.75 g of N, N'-methylene-bis-acrylamide were weighed and dissolved in 100 ml sterile water for injection. The solution was filtered through Whatman paper no.1, kept away from light and stored at 4^{0}C.

6.1.2 50% (49:1) acrylamide stock solution with 2% cross link

As described in 4.2.1.1

6.1.3 4% acrylamide with 1.5% crosslink

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4.54 ml of 50% (49.25:0.75) Acrylamide stock solution with 1.5% cross link and 0.5 ml of 10XTBE were mixed and adjusted to the total volume of 6 ml with sterile water.

6.1.4 6% acrylamide with 1.5% crosslink

0.72 ml of 50% (49.25:0.75) Acrylamide stock solution with 1.5% cross link and 0.5 ml of 10XTBE were mixed and adjusted to the total volume of 6 ml with sterile water.

6.1.5 8% acrylamide with 2% crosslink

5.02 ml of 50% (49:1) Acrylamide stock solution with 2% cross link and 0.5 ml of 10XTBE were mixed and adjusted to the total volume of 6 ml with sterile water.

6.1.6 10% acrylamide with 2% crosslink

1.2 ml of 50% (49:1) Acrylamide stock solution with 1.5% cross link and 0.5 ml of 10XTBE were mixed and adjusted to the total volume of 6 ml with sterile water.

6.1.7 20% ammonium persulfate

As described in 4.2.1.2

6.1.8 1X Loading dye

As described in 4.2.1.3

6.1.9 Formamide dye (Bromophenol blue – Xylene cyanol stock solution)

200µl of Bromophenol blue-Xylene cyanol FF solution, 800µl deionized formamide, 200 µl of 0.5 M EDTA were aliquoted. The solution was homogeneously mixed and kept at -20°C in dark.

6.2 Glass plate preparation for mini gel

The plates were cleaned with tap water and detergent and then thoroughly rinsed with distilled water to remove residual detergent. The plates were then washed with 70% ethanol. For each sandwich, one notched glass plate one rectangular glass plate and two spacers were required. The notched plate was laid on the flat surface and each spacer was placed along each edge extending along the side

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of notch. The glass plates were fit onto the spacers. The top of the T of spacers were rested along the sides of the gel sandwich. On a flat surface, the spacers were aligned with the sides and also bottom of the glass plates. The sandwich was hold into the clamp assembly, making sure the bottom edge of the sandwich was rested on the flat surface. While holding the sandwich in place, slide each clamp into position and gently tighten the screws. The bottom- edge alignment of the sandwich was checked and adjusted the glass and spacer to ensure that the bottom edges were completely flushed. The caster was standing upright or hung from the Hoefer chamber.

6.3 8% Non-denaturing polyarylamide gel preparation

1.6 milliliters of 50% (49:1) acrylamide stock solution and 0.5 ml of 10X TBE was mixed and adjusted to the total volume of 10 ml with sterile water for injection. 50 µl of 20% ammonium persulfate and 5 µl of TEMED were added and mixed gently. The gel solution mixture was immediately poured between the minigel glass plates using 1000 µl pipette until the glass plate sandwich was full. A comb was placed in the assembled gel sandwich. The gel was left to polymerize for at least 30 minutes at room temperature. The electrophoresis was pre-run for 10 min at 100 volts before samples were loading.

6.4 4%-8% gradient non-denaturing polyarylamide gel (1.5%-2%crosslink)

The glass plate was set as previously described (6.2). The gradient maker was set up with a stirrer in the downstream chamber and tubing leading from the gradient maker to the top of the gel plates. The top middle of the glass plates was attached by a yellow tip. The valve separating the two sides of the gradient maker and the tubing valve were initially close. Before pouring the gel 40µl of 20 % ammonium persulfate and 4µl of TEMED were added to both 4%and 8% acrylamide solution and the gel solution were mixed gently. The high percent gel mix was poured into the down stream chamber and low percentage gel mix into the upstream chamber of the gradient maker. The stirrer was ensured to mixing well in the downstream side. The valve between the two site of the gradient maker was then opened and also the valve in the tubing that leads to the gel plates. In the gradient maker, the 4 % gel mix flowed into the 8% gel mix forming the linear gradient of decreasing percentage concentration (in order to decrease percentage of gel from bottom to top). After the sandwich plates

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were full with gel, insert the comb without trapping small bubbles, under the wells. After pouring the gel, the gradient maker was immediately rinsed with distilled water. The comb was removed after the gel was polymerised. The electrophoresis was pre-run for 30 min at 100 volts before samples were loading.

6.5 6%-10% gradient non-denaturing polyarylamide gel (1.5%-2%crosslink)

The glass plate was set as previously described (6.2). The gradient maker was set up with a stirrer in the downstream chamber and tubing leading from the gradient maker to the top of the gel plates. The top middle of the glass plates was attached by a yellow tip. The valve separating the two sides of the gradient maker and the tubing valve were initially close. Before pouring the gel 40µl of 20 % ammonium persulfate and 4µl of TEMED were added to both 6%and 10% acrylamide solution and the gel solution were mixed gently. The high percent gel mix was poured into the down stream chamber and low percentage gel mix into the upstream chamber of the gradient maker. The stirrer was ensured to mixing well in the downstream side. The valve between the two site of the gradient maker was then opened and also the valve in the tubing that leads to the gel plates. In the gradient maker, the 6 % gel mix flowed into the 10% gel mix forming the linear gradient of decreasing percentage concentration (in order to decrease percentage of gel from bottom to top). After the sandwich plates were full with gel, insert the comb without trapping small bubbles, under the wells. After pouring the gel, the gradient maker was immediately rinsed with distilled water. The comb was removed after the gel was polymerised. The electrophoresis was pre-run for 30 min at 100 volts before samples were loading.

6.6 Procedure

SSCP was performed by the method as described by Orita et al. (13). One µl of each set of PCR products was mixed with 10 µl of 5x formamide dye mix, incubated in a boiling water-bath for 10 min before loading. Electrophoresis was carried out in 8% non-denaturing polyarylamide gel or 4%-8%, 6%-10% gradient non-denaturing polyarylamide gel and 0.5XTBE buffer without glycerol at 100 volts for 2.30-4 hour at 4^{o}C with Hoefer (MIGHTY SMALL II SE260, Amersham Biosciences, USA) electrophoresis unit. Then SSCP was visualized by silver staining (14).

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7. Silver staining

Highly sensitive detection of nucleic acids in the nanogram range has been achieved by the specific chemical reduction of silver ions. The methods for silver-staining nucleic acids of employ either a histologically derived procedure that uses ammoniacal solutions of silver, or a photochemically derived reaction in which silver binds to nucleic acid bases and is then selectively reduced by chemical agents or light. These silver staining protocols can be as sensitive as radioisotopic methods. However, they are complex and time consuming and require the preparation and handling of several solutions. In an attempt to simplify the routine use of silver stains to detect nucleic acids, Bassam *et. al.* (52) optimized the photochemically derived silver stain originally introduced by Merril *et. al.* (53) for protein staining and later applied to nucleic acids which uses formaldehyde to selectively reduce silver ions to metallic silver under alkaline conditions (41).

7.1 Reagent preparation

Reagents for silver staining method comprise three solutions, fix and stop solution (10% glacial acetic acid), staining solution, and developing solution.

7.1.1 Fix/stop solution (10% glacial acetic acid)

100 ml of glacial acetic acid was added to 900 ml of distilled water.

7.1.2 Staining solution (0.2% $AgNO_3$)

2 g of silver nitrate was weighed and homogeneously dissolved in 1 L of sterile deionized water.

7.1.3 Developing solution (3%$NaCO_3$ with 37% formaldehyde; 100ml: 150µl)

3 g of sodium carbonate was weighed and dissolved in 100 ml of sterile water for injection. The solution was chilled to 10^0 C in the refrigerator. Immediately before use, 150 µl of 37 % formaldehyde was added.

7.2 Procedure

The staining procedure consists of just a few steps. After electrophoresis, the gel frames were removed from the electrophoretic apparatus. The screws were loosening, and the plates were removed out of the frame. The glass plates were separated by spacer at a lower corner. The short glass plates adhered with the gel

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was placed in a plastic tray, fixed with 100 ml of fix solution, the glass plate was taken out and the gel in the fix solution. The gel was agitated well for 30 minutes or until the tracking dyes were no longer visible. Fixative was saved for later use in terminating the development reaction. The gel was rinsed three times in sterile water for injection with two minutes of agitation each time. After the gel was completely rinsed, it was transferred to 100 ml of staining solution and was agitated for 30 minutes. The gel was removed from the staining solution and rinsed for no longer than 5-10 seconds to remove an excess of silver nitrate in sterile water for injection. The gel was then placed in a chilled tray containing cold developing solution and was agitated again. As soon as the bands were clearly resolved, the fixative solution from the first step was poured directly into the developer, and the mixture was agitated for 3 min. The gel was rinsed twice in distilled water. The gel was collected by wrapping with cellophane membrane, and then air dried for over night at room temperature.

8. DNA sequencing

When SSCP variants were detected, the original genomic template was used for DNA sequencing analysis. The original DNA template was amplified and purified using QIAquick Gel Extraction Kit (QIAGEN, Germany), and automated DNA sequencing by automatic sequencer 3730x (DNA Sequencing Services of Macrogen Inc, Korea).

8.1 DNA template preparation

The PCR products was used as sequencing templates have to be cleaned to remove primers, nucleotides, *Taq* polymerase and salts which affect the sequencing reactions. 100 µl of PCR products were run on 1%gel electrophoresis (as described in 4.1.2.2).The DNA band was purified by using QIAquick Gel Extraction Kits. The QIAquick Gel Extraction protocol for DNA purification from agarose gels was described as followings. The DNA fragment was excised from the agarose gel with a clean, sharp scalpel. The excised DNA fragment in agarose gel was transferred to 1.5 ml microcentrifuge tube and weighed. Three volumes of Buffer QG were added to 1 voloume of gel (100 mg ~ 100 µl). An incubate was made at 50°C for 10 min or until the gel slice was dissolved completely. To assist dissolve gel, the gel mixture was mixed by vortexing the tube every 2-3 min during the incubation. One gel volume of isopropanol was then added to the sample and mixed. A QIAquick spin column was

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placed in a provided 2 ml collection tube. The sample was applied to the QIAquick column and centrifuged for 1 min at 13,000 rpm. For the gel mixture volumes of more than 800µl, the excess volume of the gel mixture was simplyed load and spun again. Flow -through was discarded and QIAquick column was placed back in the same tube. A 0.5 ml of Buffer QG was added to QIAquick column and centrifuged for 1 min at 13,000 rpm. To wash the column, 0.75 ml of buffer PE (150µl of PE and 600µl of absolute ethanol) was added to QIAquick and centrifuged for 1 min at 13,000 rpm. The flow-through was discarded and the QIAquick column was centrifuged for an additional 1 min at 13,000 rpm. The Place QIAquick column was placed into a clean 1.5 ml microcentifuge tube. To elute DNA, 30 µl of buffer EB or H_2O was added to the center of the QIAquick membrane, let stand for 1 min and then the column centrifuged for 1 min.

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CHAPTER IV
RESULTS

Multiplex assay

Multiplex assay is the assay in which several parts of gene are co-analyzed in one assay. It can be performed by two ways. First, multiplex at the PCR step and second multiplex at the SSCP step. In this study, multiplex PCR-SSCP was initially proposed to develop. Only three DNA fragment (exon 3, exon 4 and exon 13) of LDLR were performed multiplex PCR prior SSCP analysis. This multiplex PCR-SSCP technique was applied in 11 unrelated patients with primary hypercholesterolemia from Department of preventive medicines. The others DNA fragment of LDLR were not performed multiplex PCR because there were not the optimal condition for multiplex PCR. Therefore, PCR-multiplex SSCP was thus subsequently developed. Multiplex assay in this study was shown in figure 9.

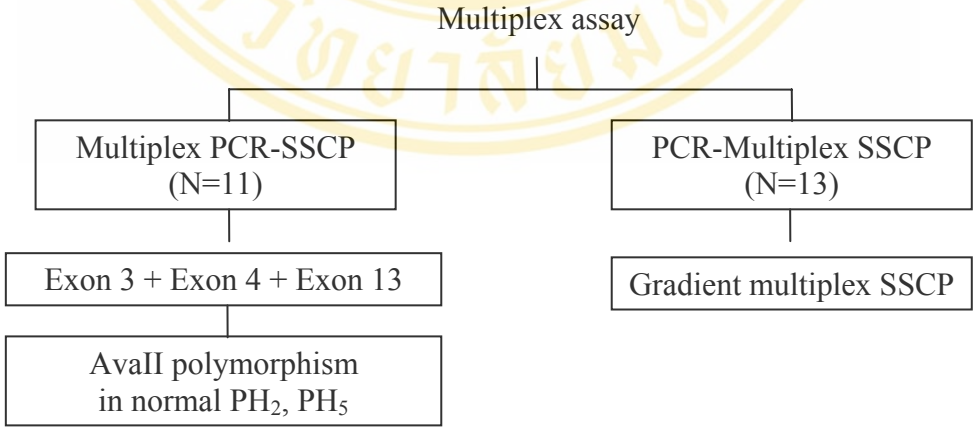

Figure 9 Multiplex assay of LDLR gene in this study

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1. Multiplex PCR assay

We have been attempting to develop a rapid, effective screening assay enabling the simultaneous analysis of DNA alteration in the LDL receptor gene. Reaction conditions, including both reagent components and cycle condition were set up to optimize the assay. For preliminary experiment, multiplex PCR-SSCP has been developed for simultaneous analysis of mutation in exons 3, 4 and 13 of LDLR gene in primary hypercholesterolemia patients. The temperature profile of 1 min at 94°C, 1 min at 49°C (annealing temperature) and 2 min at 72°C were found to be optimal to co-amplify DNA fragments of exon 3, exon 4 and exon 13 in one PCR reaction. The amplification fragment of multiplex PCR products was shown in Figure 10. One μl of multiplex PCR products was mixed with 10 μl of 5x formamide loading dye, incubated in a boiling water-bath for 10 min before loading. Electrophoresis was carried out in 8% polyacrylamide gel with 1.3%crosslink and 0.5XTBE without glycerol at 100 volts for 2.5 hour at 4^{o}C with the Mini PROTEAN II cell electrophoresis unit (BioRAD). And then SSCP gel was visualized by silver staining, wrapped by cellophane and air dried. Multiplex PCR-SSCP pattern of exons 3, 4 and 13 at the LDLR gene locus of hypercholesterolemic patients and control subject was shown in figure 11. Two identical mobility shift was observed in PH_2 and PH_5 samples. These differences were confirmed by SSCP analysis of individual exon (data not show) and further characterized by automated DNA sequencing. There were AvaII polymorphism in exon 13 of PH_2 and PH_5.

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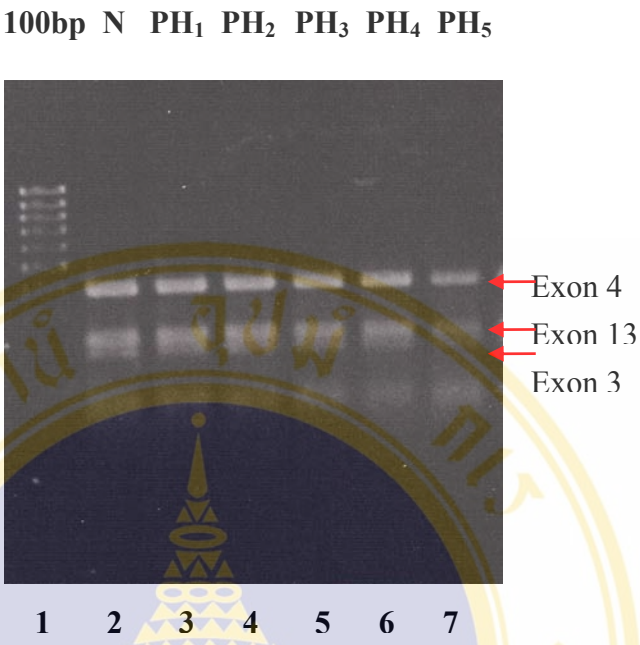

Figure 10 The amplification fragment of multiplex PCR of exons 3, 4 and 13 of LDLR gene. Lane 1 was 100 bp ladder markers. Lanes 2-7 were multiplex PCR fragments of N, PH_1, PH_2 , PH_3 , PH_4 and PH_5 , respectively.

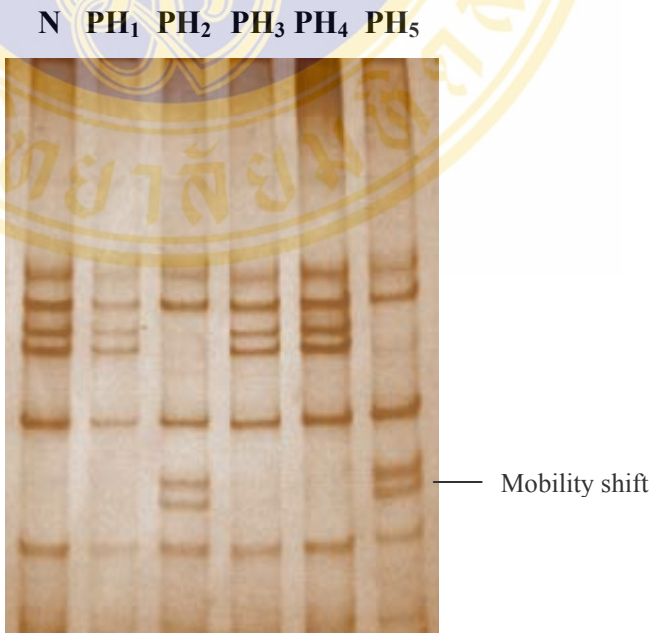

Figure 11 Multiplex PCR-SSCP pattern of exons 3, 4 and 13 at the LDLR gene locus of hypercholesterolemic patients and control subject. Electrophoresis was carried out in 8% polyacrylamide gel with 1.3%crosslink at 100 volts for 2.5 hours at 4°C.

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2. Multiplex SSCP assay

2.1 Optimum PCR-multiplex SSCP condition

PCR-multiplex SSCP was developed for diagnosis of FH. The specific PCR primers that amplified all 18 exons and the promoter region were listed in Table 5. During the design of the combination sets, we combined different migrating bands of each exon in the multiplex SSCP analysis. All PCR products of 18 exons and promoter of the LDLR gene were multiplexed into 6 combination sets. Some amplicons of the combination sets were digested with RE for two purposes. First, it was expected that exon with abnormal pattern would be specified right away from the multiplex SSCP pattern. Second, the RE digestion was expected to increase sensitivity of the multiplex SSCP. Webcutter or NEBcutter were used to identify site cut of some exons. Combination of amplified exons or RE-digested amplified exons for multiplex SSCP analysis was shown in Table 9.

Table 9 Combination of amplified exons or RE-digested amplified exons for multiplex SSCP analysis

Set	Exons in combination	Restriction Modification	
		Exon(s)	Enzyme
1	9,11,14	11	*Hae III*
2	3,4,15	3,4,15	*HpaII*
3	Promoter,1,5	Promoter	*HpaII*
4	2,6,13,18	2	*HhaI*
5	7,8,12	7	*Hae III*
6	10,16,17	-	-

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To determine the best resolution setting, we evaluated different gradient polyacrylamide gel and time. Optimal combination sets were achieved by testing under non-gradient (8%T) or gradient [4%T(1.5%C)-8%T(2%C) or 6%T(1.5%C)-10%T(1.5%C)] polyacrylamide gel electrophoresis. The gradient polyacrylamide gel was of better resolution than non-gradient polyacrylamide gel. The 4%T(1.5%C) to 8%T(2%C) gradient polyacrylamide gel electrophoresis was used in PCR-multiplex SSCP combination set 1, 2, 5, 6 and the 6%T(1.5%C) to 10%T(1.5%C) was used in PCR-multiplex combination set 2 and 4. The optimal running time was 2.30-4 hours depending on DNA fragment. The conditions for these multiplex SSCP assays were summarized in Table 10.

Table 10 The conditions for multiplex SSCP assays

Combination Set	PCR product : formamide dye (µl)	% gradient polyacrylamide gel	Running Time (hrs.)	Voltage
1	1: 5	4-8	3	100
2	1.5: 3	4-8	3	100
3	1: 3	6-10	4	100
4	1: 3	6-10	4	100
5	1.5: 3	4-8	2.30	100
6	1.5: 3	4-8	3	100

2.2 Validation of PCR-multiplex SSCP setup in LDLR gene

There were 6 combination sets of PCR-multiplex SSCP. Only 5 combination sets can be validated. They were combination sets 1, 2, 4, 5 and 6. Previously reported known mutations M412T and S554L were used to validate combination set 1. IVS 3+1 G > T (313+1G>T) was used to validate combination set 2. G1414A common polymorphism was used to validate combination set 6. The common AvaII and HincII polymorphisms can detect in exon 13 and exon 12 by PCR-multiplex SSCP combination set 4 and 5, respectively. Twenty four unrelated patients with FH were screened for mutations and polymorphisms in all 18 exons and the promoter region of the LDLR gene. We did not observe any mutation or

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polymorphism in promoter, exon 1 or exon 5 of these 13 unrelated patients with primary hypercholesterolemia from Navy Hospital. Therefore, we can not show the validation of combination set 3.

2.2.1 PCR-multiplex SSCP of the combination set 1

This combination set 1 was validated with M412T (exon 9) and S554L (exon11) mutations. The M412T mutation caused by a transition of T to C at nucleotide position 1235 in exon 9. This nucleotide change was predicted to cause the substitution of a threonine for methionine at codon 412. The amino acid at this position has been changed from an amino acid with nonpolar side chain (M) to uncharged polar side (T). The S554L mutation caused by a transition of C to T at nucleotide position 1666 in exon 11. This nucleotide change was predicted to cause the substitution of serine to leucine at codon 554. The amino acid at this position has been changed from an amino acid with uncharged polar side chain (S) to nonpolar aliphatic side chain (L). The abnormal SSCP patterns of M412T and S55L were shown below.

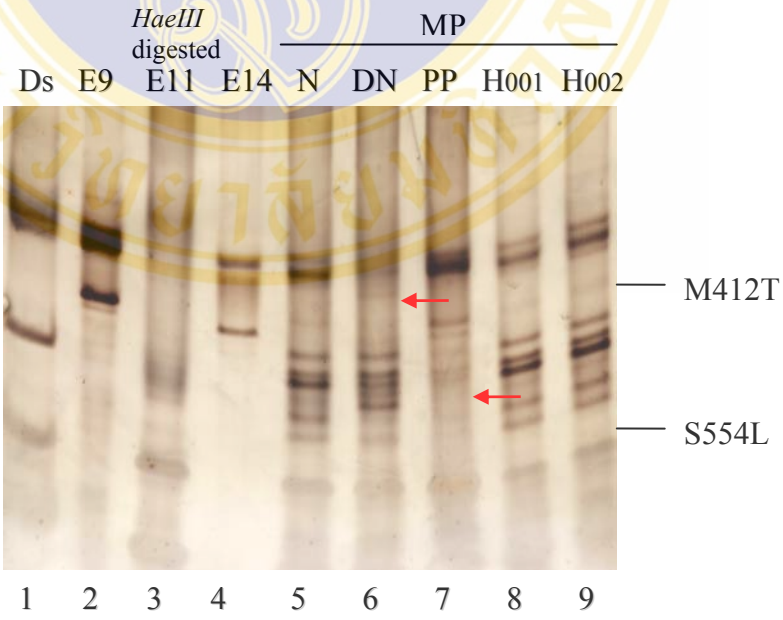

Figure 12 Silver staining of 4-8% gradient polyacrylamide gel of PCR-multiplex SSCP combination set 1. DS in lane 1 was double stranded DNA. Lanes 2-4 were individually amplified PCR fragments of exon 9, *HaeIII* digested exon 11 and exon 14 of normal control. Lanes 5-9 were fragments of PCR-multiplex SSCP of normal

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control, DN, PP, H001 and H002, respectively. DN, PP, H001 and H002 were patients with FH phenotype.

2.2.2 PCR-multiplex SSCP of the combination set 2

The combination set 2 was validated with IVS3+1G>T of exon 3. The IVS 3+1 G>T mutation caused by the G to T transversion at position 1 of intron 3. The GT dinucleotide at 5' end of an intron is highly conserved serving as part of the consensus recognition signal for mRNA splicing in vertebrate genes. The multiplex SSCP of the combination set 2 showing the abnormal SSCP pattern of IVS3+1 G>T. The data was shown in figure 13.

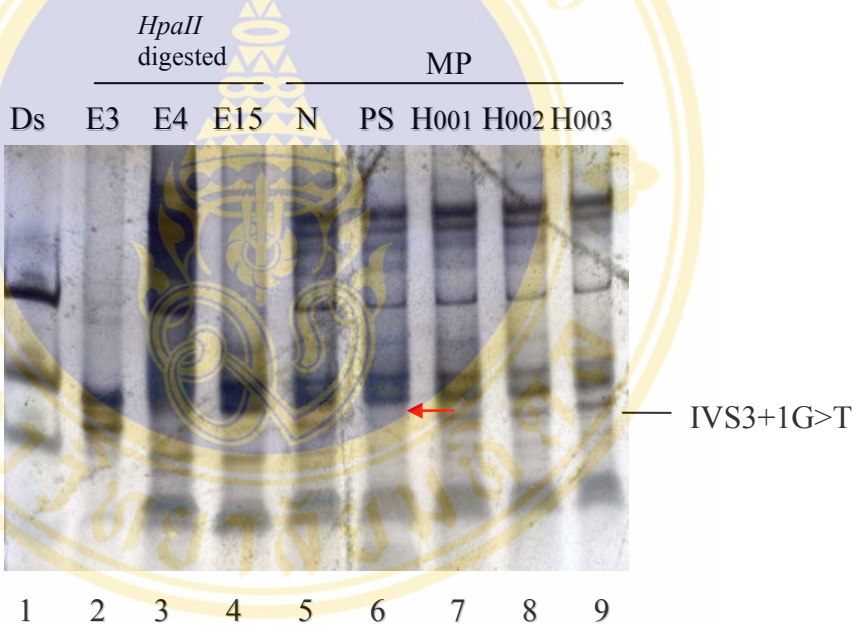

Figure 13 Silver staining of 4-8% gradient polyacrylamide gel of PCR-multiplex SSCP combination set 2. DS in lane 1 was double stranded DNA. Lanes 2-4 were individually amplified PCR fragments of *HpaII* digested exon 3, HpaII digested exon 4 and HpaII digested exon 15 of normal control. Lanes 5-9 were fragments of PCR-multiplex SSCP of normal control, PS, H001, H002 and H003, respectively. PS, H001, H002 and H003 were patients with FH phenotype.

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2.2.3 PCR-multiplex SSCP of the combination set 6

The common polymorphism G1414A in exon 10 was used for validation of this multiplex set. G225 was a 14 years old girl previously diagnosed as homozygous FH patient. This patient has homozygous A at this polymorphic site. This nucleotide alteration did not change amino acid (arginine to arginine) at codon 471 of the LDLR gene. G1414A(R471) polymorphism has been reported in NCBI database.

Figure 14 Silver staining of 4-8% gradient polyacrylamide gel of PCR-multiplex SSCP combination set 6. DS was double stranded DNA. Lanes 2-4 were individually amplified PCR fragments of exons 10, 16 and 17 of normal control. Lanes 5-7 were fragment of PCR-multiplex SSCP of normal control, G225 and H001. G225 and H001 were patients with FH phenotype.

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(A) (B)

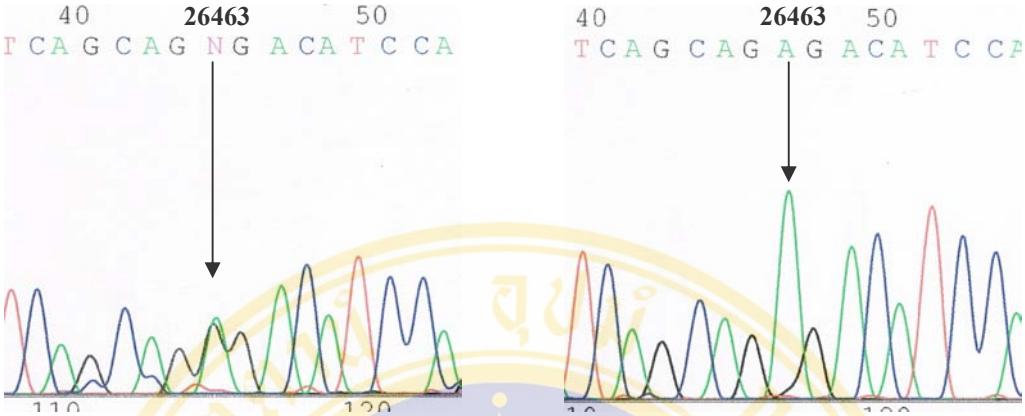

Figure 15 Nucleotide sequences from amplified exon 10 of the LDLR gene, after direct sequencing of PCR products. (A) Heterozygous for a G to A transition at nucleotide position 1414 (26463 including intron) of normal control. (B) Homozygous A at the same nucleotide position of G225 and H001.

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2.2.4 PCR-multiplex SSCP combination set 3

No mutations or polymorphisms in Exon 1, Exon 5 or promoter in our FH patients. Furthermore, we did not found mutations or polymorphisms in promoter, exon 1 or exon 5 of 24 unrelated patients with FH. Therefore, we can not show the validation of the PCR-multiplex SSCP combination set 3. Although, there were not mobility shift in this combination set but SSCP patterns were clearly and should be useful for screen mutations and polymorphisms in E1, E5 and promoter of LDLR gene.

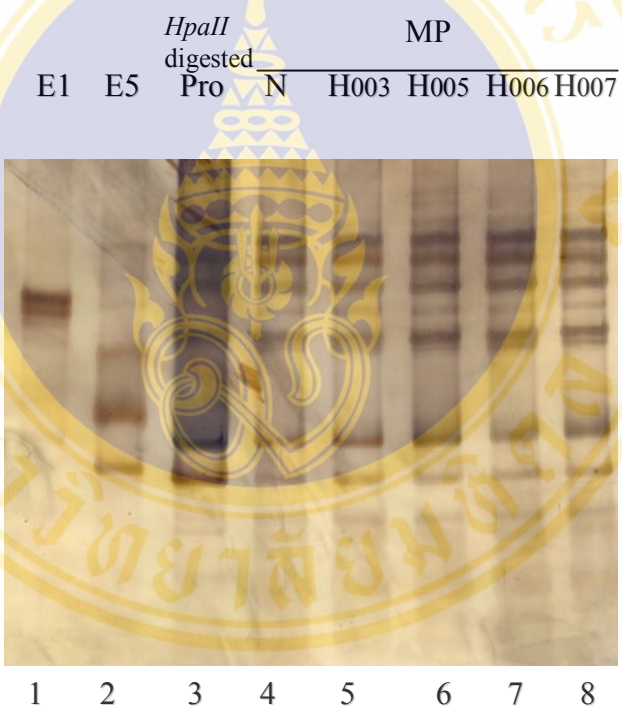

Figure 16 Silver staining of 6-10% gradient polyacrylamide gel of PCR-multiplex SSCP combination set 3. Lanes 1-3 were individually amplified PCR fragments of exon 1, exon 5 and *HpaII* digested promoter of normal control. Lanes 4-8 were fragment of PCR-multiplex SSCP of normal control, H003, H005, H006 and H007, respectiy. H003, H005, H006 and H007 were patients with FH phenotype.

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2.2.5 PCR-multiplex SSCP combination set 4

The abnormal SSCP pattern was observed in exon 13 of H007. The data have been shown as below.

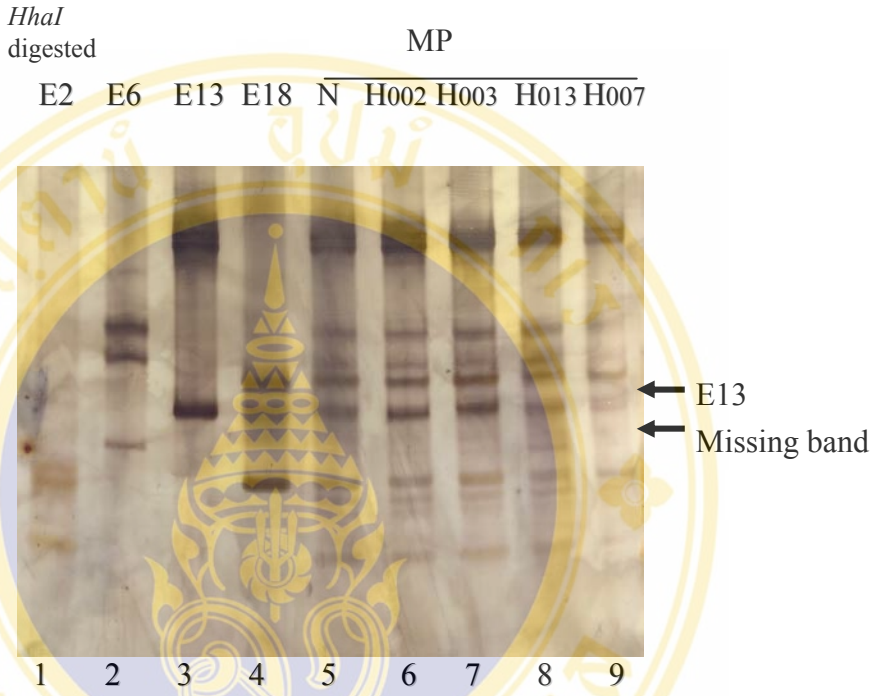

Figure17 Silver staining of 6-10% gradient polyacrylamide gel of PCR-multiplex SSCP combination set 4. Lanes 1-4 were individually amplified PCR fragments of *HhaI* digested exon 2, exon 6, exon 13 and exon 18 of normal control. Lanes 5-9 were fragment of PCR-multiplex SSCP of normal control, H002, H003, H013 and H007, respectively.

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Then sequence variations were confirmed by direct sequencing. There were heterozygous for a T to C transition of H007 at nucleotide position 33039. The nucleotide change did not disturb amino acid sequence (GTT/GTC→Val/Val). The other subjects i.e., H002, H004 and H013 were of homozygous T at this nucleotide. This nucleotide change was the common AvaII polymorphism (SNP) in exon 13 of LDLR gene. The representative sequence results have been shown as below.

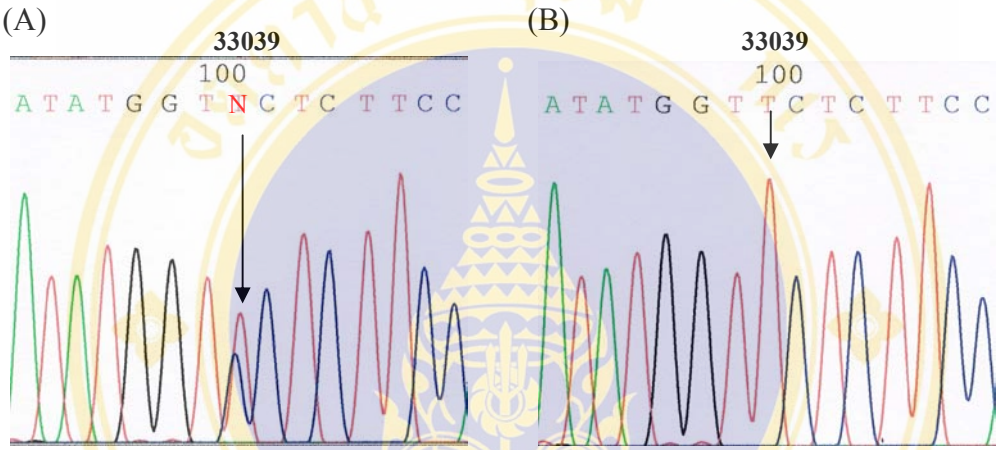

Figure 18 Nucleotide sequences from amplified exon 13 of the LDLR gene, after direct sequencing of PCR products (A) Heterozygous for a T to C transition at nucleotide position 1959 (33039 including intron) of H007. (B) Homozygous T at the same nucleotide position of H013.

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The PCR products of E13 were digested with *AvaII* to confirm the presence of this common SNP in our subjects. The amplified product of this region was 219 bp. The digestion of the amplified product with the restriction enzyme *AvaII* reveal two fragments of 182 and 37 bp, indicating the presence of the restriction site. The *AvaII* restriction map and the RFLP result were shown in figure 19.

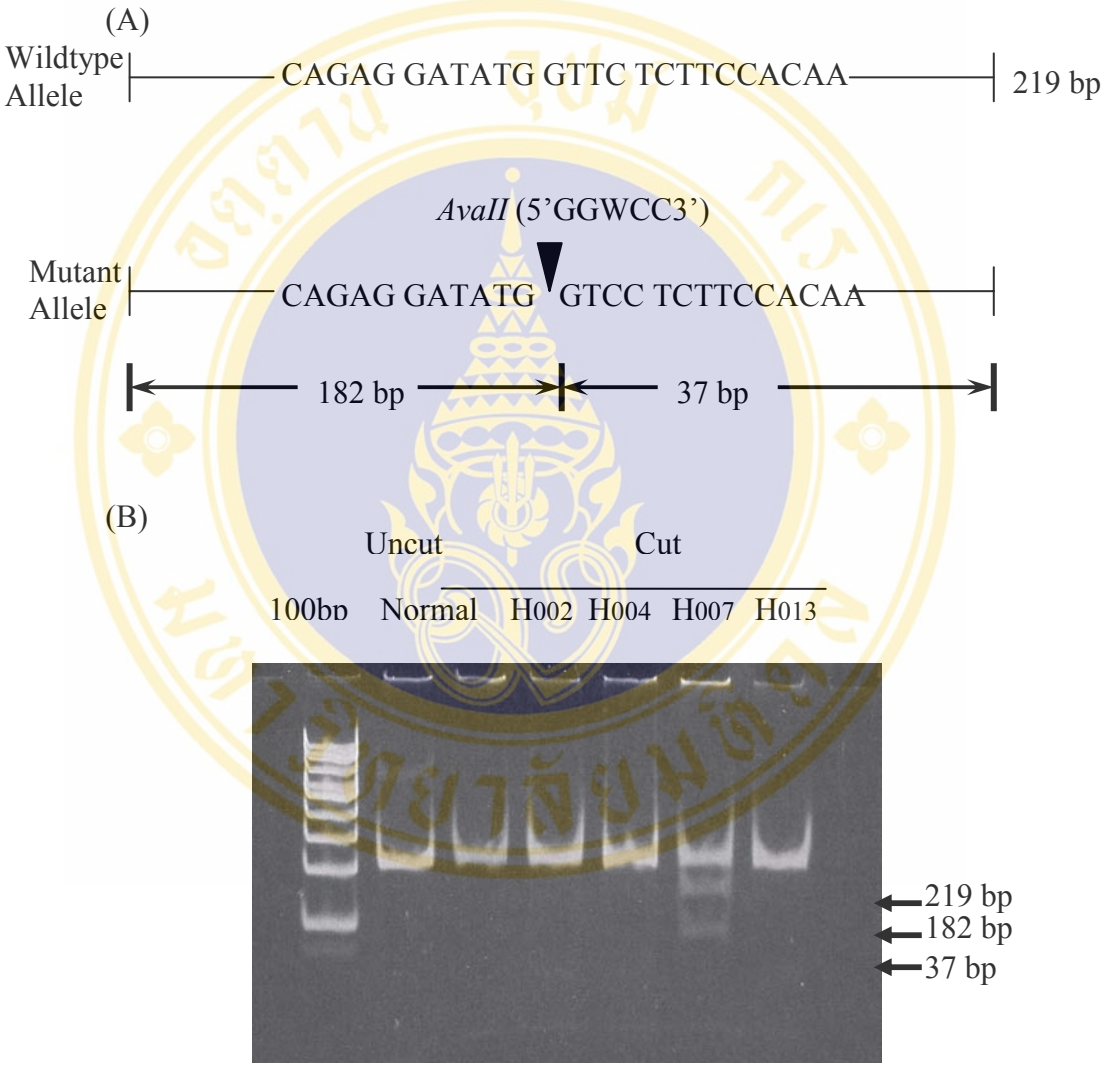

Figure 19 (A) The schematic drawing of *AvaII* digestion site in the PCR product of exon 13 of LDLR gene. (B) Agraose gel electrophoresis of AvaII polymorphism in exon 13 of the LDLR gene.

From RFLP result, we conclude that H007 was heterozygous for T to C transition and the other subjects i.e., H002, H004 and H013 were homozygous T corresponding with the DNA sequence data. This AvaII polymorphism was recorded

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in the LDLR database (Appendix B). The allele specific frequency of this polymorphism of C is 0.37.

2.2.6 PCR-multiplex combination set 5

The PCR-multiplex SSCP gel of combination set 5 was shown in figure 16. We found that H004 had the same SSCP pattern as normal control, H001was the same as H002 and H003 was different from the others. The mobility shifts were apparent in exons 8 and 12. Therefore, exons 8 and 12 of these DNA samples were subjected to DNA sequencing.

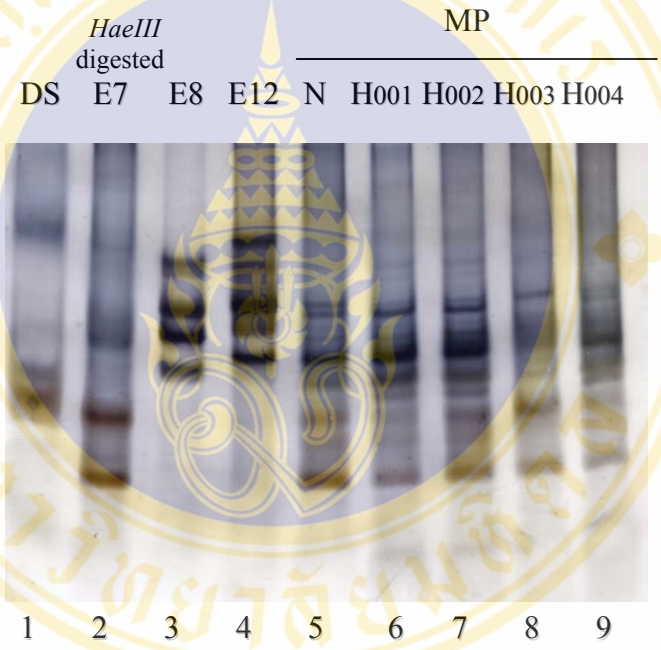

Figure 20 Silver staining of 4-8% gradient polyacrylamide gel of PCR-multiplex SSCP combination set 5. DS in lane 1 was double stranded DNA. Lanes 2-4 were individually amplified PCR fragments of *HaeIII* digested exon 7, exon 8 and exon 12 of normal control. Lanes 5-9 were fragment of PCR-multiplex SSCP of normal control, H001, H002, H003 and H004 respectively. H001, H002, H003 and H004 were patients with FH phenotype.

From DNA sequencing results of E8, there were heterozygous for a G to A transition at nucleotide position 24494 in normal control, H001, H002, H003 and H004. The nucleotide change did not disturb amino acid sequence (GAA/AAA→Lys/Lys). It might be novel polymorphism. In exon 12, there were

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heterozygous for a C to T transition at nucleotide position 29800 in H003 and homozygous C in normal control, H001, H002 and H004. The nucleotide change did not disturb amino acid sequence (AAC/AAT→Asn/Asn) as well. Previous study in our lab shows that this is HincII polymorphism.

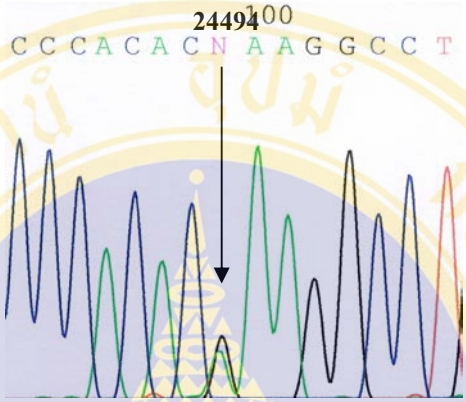

Figure 21 Nucleotide sequence from amplified exon 8 of the LDLR gene, after direct sequencing of PCR products. Heterozygous for a G to A transition at nucleotide position 1167 (24494 including intron).

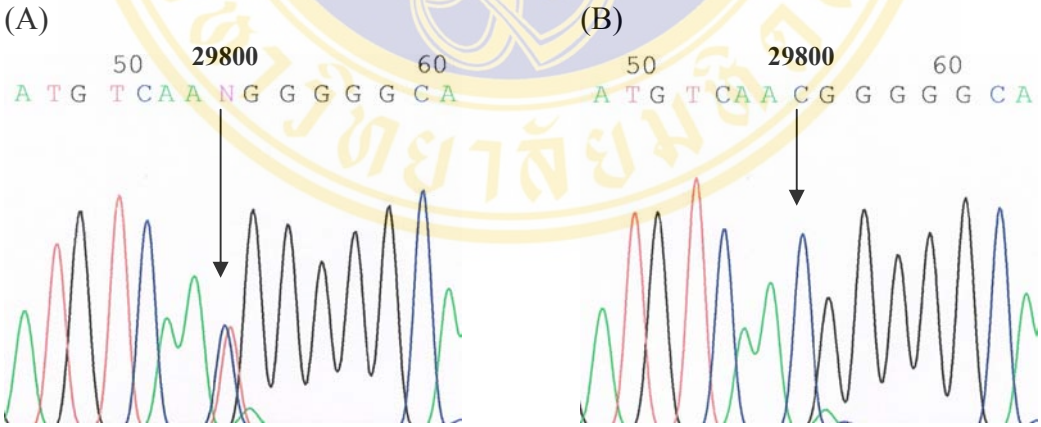

Figure 22 Nucleotide sequence from amplified exon 12 of the LDLR gene, after direct sequencing of PCR products. (A) Heterozygous for a C to T transition of H003 of nucleotide position 1773 (29800 including intron) (B) Homozygous C of normal control.

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CHAPTER V
DISCUSSION

In most populations in the world, the frequency of heterozygous FH is estimated to be 0.2%. At the molecular level, FH is a heterogeneous disorder caused by more than 900 different mutations in the LDLR gene and the number of identified mutations is increasing steadily (4). Identification of a LDL receptor gene mutation in an FH individual is of value for several reasons. An unequivocal diagnosis can be made, and affected relatives can be identified at an early age, when a clear-cut diagnosis based on clinical criteria is not possible. Mutation detection can be divided into two categories: a scanning mode, where a stretch of DNA is searched for unknown mutations, and diagnostic mode, where specific tests are designed to detect known mutations (42). To answer the questions in a genetically heterogeneous population, new molecular genetic techniques had to be established that allow a rapid and sensitive screening for new mutations. SSCP analysis of PCR-amplified genomic DNA fragments (Orita *et al* 1989) has become a simple and sensitive standard method for the detection of small genetic alteration. We further modified this technique by performing PCR-multiplex SSCP. In this study, we devised a PCR-multiplex SSCP to reduce the workload involved in performing mutation analysis in large regions of LDLR gene. The protocols were validated via known mutations or polymorphisms. In the analysis of DNA segments of which no genetic variation existed, validation was made by performing DNA sequencing when abnormal SSCP pattern or mobility shift was apparent.

Multiplex assay is the assay in which several parts of gene are co-analyzed in one assay. It can be performed by two ways. First, multiplex at the PCR step: more than one primer pairs were simultaneously used in one PCR reaction mixture prior to SSCP analysis. Pocsai *et al* developed multiplex PCR assay for screening deletions in the LDLR in 2001 (1). In this study, multiplex PCR and subsequently followed by SSCP was initially tried. However, it did not work because in multiplex PCR

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condition, primers of some exons co-amplified with other part of gene so non-specific PCR product was apparent. For successful multiplex PCR assay, the relative concentration of primers, PCR buffer, balance between $MgCl_2$ and dNTP, cycling temperature, amount of DNA template and *Taq* DNA polymerase are important (43). Second, multiplex at the SSCP step: PCR for individual exons or for individual part of the gene were performed and then these PCR products were pooled in multiplex assay by SSCP. Previous study, the former students used both techniques in the EGF precursor homology domain (E7-E14) and the ligand binding domain (E2-E6) of the LDLR gene (48, 49). In this study, multiplex PCR-SSCP was initially proposed to develop. After several trials of multiplex PCR-SSCP upon various combinations of amplified exons, only the combination of exons 3, 4 and 13 of LDLR gene gene was successfully optimized (as presented in Fig. 10). Therefore, PCR-multiplex SSCP was thus subsequently developed. The development was fairly successful and the protocol was applicable to investigate the whole regions of LDLR gene (promoter plus 18 exons). PCR fragments of some amplified exons were digested with restriction enzymes to increase sensitivity of the multiplex assay. There were 6 combination sets of amplified exons or RE-digested amplified exons for multiplex SSCP analysis. Several factors such as temperature, pH, running time, gel composition and size of the DNA fragments can influence the sensitivity of SSCP analysis (35). Many modifications to the original protocol developed by Orita *et. al.* include variables that affect the gel matrix, e.g., percentage of acrylamide monomer, cross-linking ratio, buffer systems, addition of neutral compounds to the gel and electrophoresis temperature. The most preferred gel characteristics for successful differential separation of single strand conformers in the range of 200-300 bp, where the method should be most sensitive and reliable, are 12% acrylamide and cross-linking ratios (%C) between 1 and 3 (10). Several publications have detailed the different effects that different conditions can have on resolving a particular sequence variation. Reduced cross linker ratio (bis:acrylamide 1:49) and 50-100 ml/l glycerol are popular (44). The sensitivity of the multiplex SSCP was also increased by using gradient polyacrylamide gel electrophoresis. To determine the best resolution setting, different gradient polyacrylamide gel and running time were evaluated. For PCR-multiplex

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SSCP, the gradient [4%T(1.5%C)-8%T(2%C) or 6%T(1.5%C)-10%T(1.5%C)] polyacrylamide gel revealed better resolution than non-gradient (8%T) polyacrylamide gel. The optimal running time of these multiplex SSCP in gradient gel was 3-4 hours depending on DNA fragments. So far no publication reported gradient acrylamide gel electrophoresis in SSCP analysis. This study in our laboratory is thus the first report on multiplex SSCP analysis for mutation screening at the LDLR gene locus.

The sensitivity of a mutation detection method has usually been evaluated by the rate of detection of already known mutation (35). We have demonstrated the use of PCR-multiplex SSCP method for detection of M412T (exon 9) and S554L (exon 11) in combination set 1, IVS 313+1 G > T (exon 3) in combination set 2 and G1414A (exon 10) common polymorphism in combination set 6. In the combination set 1 (figure 9), abnormal mobility shifts of DN and PP samples were observed. Mobility shifts in exon 9 of M412T mutation and in exon 11 of S554L mutation were observed in the corresponding exonic banding patterns of the multiplex SSCP set 1. In the combination set 2 (figure 10), abnormal mobility shift of PS sample was observed. This mobility shift was correspondingly apparent in the region of banding pattern of exon 3. In the combination set 6 (figure 11), the multiplex SSCP banding pattern of G1414A (exon 10) in normalipidemic subject was apparently different from the banding pattern of homozygous A in FH patients (G225 and H001). For the multiplex SSCP in the combination set 3 (figure 13), there was no genetic variation for validation at present time. However, the profile of this combination set 3 was fairly clear. It should have revealed the mobility shift pattern for any genetic variation if it is existing in any DNA segment of this multiplex set and it should thus be applicable for screening mutations and polymorphisms in exon 1, exon 5 and promoter of the LDLR gene. On the other hand, the profiling of these DNA segments can be collected as genetic profile for each FH patient. The common AvaII (exon 13) and HincII (exon12) polymorphisms could be detected by PCR-multiplex SSCP combination set 4 and 5, respectively. In combination set 4 (figure 14), the heterozygous T to C transition in exon 13 is the common AvaII single nucleotide polymorphism (SNP). It is the variation at nucleotide position 33039 with an allele frequency of C is 0.37(Appendix B). Previous study, the genotype frequencies at AvaII sites of Thai population were

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57.9%, 36.6% and 5.5% for the (-/-), (+/-), and (+/+) genotypes, repectively (50). In combination set 5 (figure 17), the heterozygous for C to T transition in exon 12 is the common HincII polymorphism (SNP). In addition, there was a heterozygous G to A transition at nucleotide position 24494 in exon 8. This polymorphism did not change amino acid (GAA/AAA→Lys/Lys) and it has not been reported elsewhere. From LDLR database (http://www.ucl.ac.uk/fh/), only StuI polymorphism in exon 8 has been reported. Therefore, this G→A transition is a novel polymorphism observed via PCR-multiplex SSCP analysis in this study.

In this study, we tried to optimize PCR multiplex-SSCP condition and used known mutation to validate this technique. The optimized PCR-multiplex SSCP were subsequently applied in 13 unrelated patients with primary hypercholesterolemia. The multiplex SSCP screening was performed for all 18 exons and promoter region in these patients. The mutations and polymorphisms used for validation of the PCR-multiplex SSCP assays and the genetic variations observed by the multiplex SSCP assays were summarized in Table 11.

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Table 11 Mutations and Polymorphisms in 13 unrelated patients with primary hypercholesterolemia using PCR-multiplex SSCP technique.

PCR-multiplex SSCP of the combination set	Exon	Change in nucleotide sequence	Nucleotide No.	Change in amino acid sequence	Mutation name
1	9	ATG>ACG	1235	Met/Thr	M412T
	11	TCG>TTG	1666	Ser/Leu	S554L
	14	-	-	-	-
2	3	IVS 3+1 G>T	313	----------	313+1 G>T
	4	-	-	-	-
	15	-	-	-	-
3	1	-	-	-	-
	5	-	-	-	-
	promoter	-	-	-	-
4	2	-	-	-	-
	6	-	-	-	-
	13	GTT/GTC	1959	Val/Val	AvaII polymorphism[a]
	18	-	-	-	-
5	7	-	-	-	-
	8	GAA/AAA	1167	Lys/Lys	Novel polymorphism[b]
	12	AAC/AAT	1773	Asn/Asn	HincII polymorphism[c]
6	10	AGG/AGA	1414	Arg/Arg	G1414A[d]
	16	-	-	-	-
	17	-	-	-	-

a= Heterozygous T/C in patient H007, Homozygous TT in patients H002, H004, H013
b= Heterozygous G/A in normal control and patients H001, H002, H003, H004
c= Heterozygous C/T in patient H003, Homozygous CC in normal control and patients H001, H002, H004
d= Heterozygous G/A in normal control, Homozygous AA in patients G225 and H001

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The patients under this study were primarily tested for the ApoB common R3500Q mutation prior to the LDLR gene screening. These patients have not been screened for the mutations in the PCSK9 gene (45) which also causes FH-like phenotype. Although new and recurrent mutations were not found in these patients, the polymorphisms observed in this study should be useful. In the general population, LDL receptor gene polymorphisms have been shown to be associated with variations in cholesterol levels (46) as well as a wide variation in the age of onset and the expression of coronary heart disease in FH patients (47).

SSCP analysis is reported to have a sensitivity of 75-85%, suggesting that a mutation may have been missed for technical reasons in 15-25% patients. This technical problem should be partly solved by trying the other optimal conditions. In case that several optimal SSCP conditions fail to reveal any mobility shift or mutation, the patients may be deserved to search for large insertion, deletion or large rearrangement by other appropriate techniques. SSCP is suitable for screening only one base change or small deletion or insertion.

The multiplex SSCP method developed in this study can considerably reduce cost (such as chemical reagents for polyacrylamide gel preparation, staining solution, etc.) and time involved in mutation detection by more than 50%. The availability of new methods that speed up the process of finding genetic aberrations in genes should allow the identification of an ever-increasing number of LDLR gene variants that cause hypercholesterolemia.

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CHAPTER VI
CONCLUSION

For establishing a molecular diagnosis, the whole LDL receptor gene of each individual patient has to be screened for new and recurrent mutations. The 6 combination sets of PCR-multiplex SSCP analysis developed in this study seem to be a suitable method. It is relatively rapid, simple, sensitive and has been successfully applied for known mutation detection. The comparison between original SSCP method (51) and PCR-multiplex SSCP in this study was summarized in Table 12.

Table 12 Comparison of original SSCP method (Orita *et. al*.) and PCR-multiplex SSCP developed in this study.

Method / Property	Original SSCP	PCR-multiplex SSCP
Rapid	- screening 1 DNA fragment/ lane/SSCP gel	- screening 3 or 4 DNA fragments/lane/SSCP gel (3-4 times more rapid)
Simple	- large gel (20×40×0.2 cm) - hybridization detection (using ^{32}P-labled DNA probes→cumbersome, dangerous and expensive)	- minigel (10×10.5×0.075 cm) (easy to handle) - detection by silver staining (avoid hazardous radioactive, inexpensive)
Sensitive	- restriction endonuclease	- restriction endonuclease in hypermutable CG region (increase sensitivity of multiplex SSCP gel, expect to find new mutation at CG region) - gradient SSCP gel

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Table 12 (Cont.) Comparison of original SSCP method (Orita *et. al*.) and PCR-multiplex SSCP developed in this study.

Method / Property	Original SSCP	PCR-multiplex SSCP
		(increase sensitivity and resolution)

The new technique has a great potential for mutation screening of large numbers of samples in clinical diagnosis. We can use this multiplex assay to screen mutation and polymorphism in DNA sample and can also create a DNA profile for an individual subject. At an individual level, when an individual tested positive for a mutation, we can use that mutation to screen his or her family members so that preventive treatment for the family members at risk can be made and/or therapeutic intervention for patient can be managed in the right direction. At population level, identification of mutations in our population will generate a database. Availability of such database for LDLR gene mutations can likely lead to an implementation for population-based genetic screening program such as those programs established in Scotland and Netherland (18). Now, CAD is the leading cause of death in many countries, including Thailand. In the future by the year 2020, CAD is predicted by WHO to be a major cause of death for global population, especially in developing countries. So if we have our own mutation database, we can use possibly this database for diagnosis and prevention in our population.

In conclusion, this knowledge will be integrated in our routine molecular diagnostic strategy for FH. This should simplify diagnostic testing, facilitate genetic counseling and make it possible to diagnose FH at the molecular level at a presymptomatic early age.

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REFERENCES

1. Pocsai Z, Paragh G and Adany R. Multiplex PCR assay for screening deletions in the low density lipoprotein receptor gene. Clin Chim Acta. 2001;309(1):7-12.
2. Liguori R, Argiriou A and Simone VD. A rapid method for detecting mutations of the human LDL receptor gene by complete cDNA sequencing. Mol Cell Probes. 2003;17(1):15-20.
3. Melissa A. Austin , Carolyn M. Hutter, Ron L. Zimmern and Steve E. Humphries. Familial Hypercholesterolemia and Coronary Heart Disease: A HuGE Association Review**.** American Journal of Epidemiology. 2004;160(5): 421-429.
4. Weiss N, Binder G and Keller C. Mutations in the low-density-lipoprotein receptor gene in German patients with familial hypercholesterolaemia. J Inherit Metab Dis. 2000;23(8):778-90.
5. Khoo KL, van Acker P, Defesche JC, Tan H, van de Kerkhof L and Heijnen-van Eijk SJ *et al.* Low-density lipoprotein receptor gene mutations in a Southeast Asian population with familial hypercholesterolemia. Clin Genet. 2000;58(2):98-105.
6. Bodamer OA, Bercovich D, Schlabach M, Ballantyne C, Zoch D and Beaudet AL. Use of Denaturing HPLC to Provide Efficient Detection of Mutations Causing Familial Hypercholesterolemia. Clinical Chemistry. 2002;48(11):1913–1918.
7. Bunn CF, Lintott CJ, Scott RS and George PM. Comparison of SSCP and DHPLC for the Detection of LDLR Mutations in a New Zealand Cohort. Hum Mutat. 2002;19(3):311.
8. Steve E. Humphries, Vilmundur Gudnason, Ros Whittall and Ian N.M. Day. Single-strand conformation polymorphism analysis with high throughput modifications, and its use in mutation detection in familial hypercholesterolemia. Clinical Chemistry. 1997; 43(3):427–435.
9. Weiss N, Eggersdorfer I and Keller C. Multiplex-PCR-based single-strand conformation polymorphism protocol for simultaneous analysis of up to five

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fragments of the low-density-lipoprotein receptor gene. Biotechniques. 1996;20(3):421-4, 426, 428-9.

10. Arseni Markoff, Alex Savov, Vladimir Vladimirov, Nadia Bogdanova, Ivo Kremensky and Varban Ganev. Optimization of single-strand conformation polymorphism analysis in the presence of polyethylene glycol. Clinical Chemistry. 1997; 43(1):30–33.
11. P. Sunnucks, A. C. C. Wilson, L. B. Beheregaray K. Zenger, J. French and A.C. Taylor. SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. Molecular Ecology. 2000;9:1699–1710.
12. Lachtermacher MB, Seuanez HN, Moser HW and Smith KD. One-step multiplex PCR strategy for identification of mutations by SSCP and DNA sequencing. Biotechniques. 2000; 29(2):234-6.
13. Orita M, Suzuki Y, Sekiya T and Hayashi K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics. 1989;5(4):874-9.
14. Pongrapeeporn KS, Thepsuriyanon P, Leowattana W, Ong-ajyooth S, Kiartivich S and Yamwong P. Inexpensive, rapid and convenient PCR-minigel SSCP protocol for polymorphisms and mutations analyses of LDL receptor gene. J Med Assoc Thai. 2001;84(3):676-83.
15. Hannna Kankkonen. Gene Therapy in The treatment of Familial Hypercholesterolemia [webpage on the internet]. Available from: http://www.uku.fi/vaitokset/2004/isbn951-781-382-1.pdf
16. Aldons J. Lusis, Rebecca Mar and Paivi Pajukanta1. Genetics of atherosclerosis. Annu. Rev. Genomics Hum. Genet. 2004;5:189–218.
17. Specialized investigations:Clinical disorders of lipid metabolism. Robert Britton. An illustrated colour text clinical biochemistry. New york:Churchill livingstone; 1995.p122-123.
18. Melissa A. Austin, Carolyn M. Hutter, Ron L. Zimmern and Steve E. Humphries. Genetic Causes of Monogenic Heterozygous Familial Hypercholesterolemia: A HuGE Prevalence Review. Am J Epidemiol. 2004;160(5):408-420.

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19. Dalya Marks, Margaret Thorogood, H. Andrew W. Neil and Steve E. Humphries. A review on the diagnosis, natural history, and treatment of familial hypercholesterolaemia. Atherosclerosis. 2003;168:1-14.

20. Varret M, Rabes JP, Saint-Jore B, Cenarro A, Marinoni JC and Civeira *et al.* A third major locus for autosomal dominant hypercholesterolaemia maps to 1p34.1-p32. Am J Med Genet. 1999;64:1378-87.

21. Hunt SC, Hopkins PN, Bulka K, McDermott MT, Thorne TL and Wardell BB *et. al.* Genetic localization to chromosome 1p32 of the third locus for familial hypercholesterolemia in a Utah kindred. Arterioscler Thromb Vasc Biol. 2000;20:1089-93.

22. Paul N. Hopkins. Familial hypercholesterolemia—Improving treatment and meeting. International Journal of Cardiology. 2003;89:13–23.

23. Philip J. Lee. The management of familial hypercholesterolaemia in childhood. Current Paediatrics. 2002;12:104-109.

24. Michael s. Brown and Joseph L. Goldstein. A receptor-mediated pathway for cholesterol homerostasis [webpage on the Internet]. Available from: http://nobelprize.org/medicine/laureates/1985/brown-goldstein-lecture.pdf

25. Department of Biology, Lake Forest College, USA. Hypercholesterolemia: So much cholesterol, so many causes [webpage on the Internet]. Available from: http://campus.lakeforest.edu/~debburman/BIO221S02/fiveprojects/diseasereviewarticle/abstracts/FH.pdf

26. A K Soutar and BL Kniht. Structure and regulation of the LDL-receptor and its gene. Lipids and cardiovascular disease. British Medical Bulletin:1990;No.4,p.891-916.

27. Mathilde Varret, Jean-Pierre Rabès, Gwenaëlle Collod-Béroud, Claudine Junien and Catherine Boileau. Software and database for the analysis of mutations in the human LDL receptor gene. Nucleic Acids Research. 1997;25(1):172-180.

28. Mathilde Varret, Jean-Pierre Rabés, Rochelle Thiart, Maritha J. Kotze, Heike Baron and Ana Cenarro *et al.* LDLR Database (second edition): new additions to the database and the software, and results of the first molecular analysis. Nucleic Acids Research. 1998;26(1):248–252

Copyright by Mahidol University

29. S.E. Humphries, D. Galton and P. Nicholis. Genetic testing for familial hypercholesterolaemia: practical and ethical issues. Q J Med. 1997; 90:169–181 [review].
30. RGH Cotton. Mutation detection. In: David N Cooper, editors. Nature encyclopedia of the human genome, Volume 4. New York: Nature Publishing Group; 2003.p.204-210.
31. Gene structure and function [webpage on the Internet]. Available from: http://www.oup.co.uk/pdf/0-19-856494-5.pdf
32. Maruyama T, Miyake Y, Tajima S, Harada-Shiba M, Yamamura T and Tsushima M *et al.* Common Mutations in the Low-Density-Lipoprotein–Receptor Gene Causing Familial Hypercholesterolemia in the Japanese Population. Arterioscler Thromb Vasc Biol. 1995;15(10):1713-8.
33. S.E. Humphries, V. Gudnason, R. Whittall and I.N. Day. Single-strand conformation polymorphism analysis with high throughput modifications, and its use in mutation detection in familial hypercholesterolemia. International Federation of Clinical Chemistry Scientific Division: Committee on Molecular Biology Techniques, Clin Chem. 1997;43(3):427–435 [review].
34. Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.gov/OMIM (for FH [143890], LDLR gene [606945]).
35. Jensen HK, Jensen LG, Hansen PS, Faergeman O and Gregersen N. High sensitivity of the single-strand conformation polymorphism method for detecting sequence variations in the low-density lipoprotein receptor gene validated by DNA sequencing. Clin Chem. 1996;42(8 Pt 1):1140-6.
36. Jensen HK. The molecular genetic basis and diagnosis of familial hypercholesterolemia in Denmark. Dan Med Bull. 2002;49(4):318-45.
37. Viroj Wiwanitkit, MD, Danai Wangsaturaka, MD and Oranee Tangphao, MD. LDL-cholesterol lowering effect of a generic product of simvastatin compared to simvastatin (Zocor™) in Thai hypercholesterolemic subjects – a randomized crossover study, the first report from Thailand. BMC Clin Pharmacol. 2002;2: 1-6.

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38. Amersham Biosciences [homepage on the Internet]. Available from : http://www.mdyn.com/aptrix/upp00919.nsf/Content/elpho_applications%5Celpho_applications_fragment_analysis%5Celpho_applications%5C2.+SSCP

39. Pluthero FG. Rapid purification of hight-activity Taq DNA polymerase. Nucleic Acids Research. 1993;(21):4850-51.

40. Durrington PN. Lipids and their metabolism: In: hyperlipidaemia; diagnosis and management, 2th ed, UK: University of Manchester; 1995.p.4-71.

41. Uni-graz.at [homepage on the Internet]. Available from : http://www.uni-graz.at/~binder/thesis/node73.html

42. Jensen HK, Jensen LG, Meinertz H, Hansen PS, Gregersen N and Faergeman O. Spectrum of LDL receptor gene mutations in Denmark: implications for molecular diagnostic strategy in heterozygous familial hypercholesterolemia. Atherosclerosis. 1999;146(2):337-44.

43. Markoulatos P, Siafakas N and Moncany M. Multiplex polymerase chain reaction: a practical approach. J Clin Lab Anal. 2002;16(1):47-51.

44. Spinardi L, Mazars R and Theillet C. Protocols for an improved detection of point mutations by SSCP. Nucleic Acids Res 1991;19:4009.

45. Sozen MM, Whittall R, Oner C, Tokatli A, Kalkanoglu HS and Dursun A *et al.* The molecular basis of familial hypercholesterolaemia in Turkish patients. Atherosclerosis. 2005;180(1):63-71.

46. Ahn YI, Kamboh MI and Aston CE *et al.* Role of common genetic polymorphisms in the LDL receptor gene in a¡ecting plasma cholesterol levels in the general population. Arterioscler Thromb. 1994;14: 663-670.

47. Hill JS, Hayden MR and Frohlich J *et al.* The incidence of coronary artery disease in heterozygous familial hypercholesterolemia. Arterioscler Thromb. (1991);11: 290-297.

48. Lampoon Kasemsuk. Multiplex SSCP analysis of EGF precursor homology domain of LDL receptor gene. [M.S. Thesis in Biochemistry]. Bangkok, Faculty of Graduates studies, Mahidol University; 2002.

49 Kwandao Kerdsaeng. PCR-minigel SSCP analysis of the exons encoding the ligand binding domain of LDLR gene. [M.S. Thesis in Biochemistry]. Bangkok, Faculty of Graduates studies, Mahidol University; 2003.

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50. Theerasak Pimsawat. Polymorphisms at the 5'flanking region, exons 1,8,13 and 18 of low density lipoprotein receptor gene in Thai subjects. [M.S. Thesis in Biochemistry]. Bangkok, Faculty of Graduates studies, Mahidol University; 1999.

51. Orita M, Iwahana H and Kanazawa H. *et. al*. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphism. Proc. Natl. Acad. Sci. USA. 1989;86:2766-2670.

52. Bassam, B. J., Caetano-Anolles, G., Gresshoff, P. M. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem.1991;195:80-83.

53. Merril, C. R., Goldman, D., Sedman, S. A., Ebert, M. H. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science. 1981;211:1437-1438.

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APPENDIX

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APPENDIX

A. Taq DNA polymerase preparation

A.1 Reagents Preparation

A.1.1 LB broth

5 g of Bacto-tryptone, 2.5 g of yeast extract and 2.5 g of NaCl were weighed and the volume was adjusted to 500 ml with sterile deionized water. The solution was sterilized by autoclaving.

A.1.2 LB agar (LB broth +1.5% agar)

3 g of the agar was weighed and dissolve in 200 ml of the LB broth.

A.1.3 Ampicillin stock (10 mg/ml)

0.1 g of ampicillin was weighed and dissolved in 1ml of autoclaved sterile water.

A.1.4 50 mM $CaCl_2$ and 10 mM Tris-HCl, pH 8.0 solution

200 µl of 1 M $CaCl_2$ and 40 µl of 1M of Tris-HCl, pH 8.0 were aliquoted. The volume was adjusted to 4 ml of autoclaved sterile water.

A.1.5 SOB solution

2 g of Bacto-tryptone, 0.5 g of yeast extract and 0.005 g of NaCl were weighed and the volume was adjusted to 100 ml with sterile deionized water. The solution was sterilized by autoclaving. The solution was stored at 4 ^{0}C until used.

A.1.6 1 M glucose

1.8 g of glucose was weighed and dissolved in 10 ml of autoclaved sterile water. The solution was filtered by 0.2 µm Nalgene filter.

A.1.7 1M $MgSo_4$

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24.65 g of $MgSo_4$ was weighed and dissolved in sterile deionized water and the volume was adjusted to 100 ml. The solution was filtered by 0.2 μM Nalgene filter.

A.1.8 SOC solution

200 μl of 1 M $MgSO_4$ and 200 μl of 1M glucose were aliquoted into 10 ml of SOB. The solution was mixed and stored at 4 ^{0}C until used.

A.1.9 Buffer A solution

1 ml of 1M Tris-HCl (pH8), 2 ml of 1 M glucose and 80 μl of 0.5 M EDTA were aliquoted and diluted in sterile deionized water. The volume was adjusted to 40 ml of autoclaved sterile water.

A.1.10 Prelysis buffer

0.004 g of lysozyme was weighed and dissolved in 10 ml of buffer A.

A.1.11 1 M KCl

7.455 g of KCl was weighed and dissolved in sterile deionized water and the volume was adjusted to 100 ml. The solution was sterilized by autoclaving.

A.1.12 100 mM PMSF

0.0174 g of PMSF was weighed and dissolved in 1ml of absolute ethanol. The solution was stored at –20 °C until use.

A.1.13 Lysis buffer

100 μl of 1 M Tris-HCl (pH 8.0), 500 μl of 1 M KCl, 20 μl of 0.5 M EDTA, 100 μl of 10 mM PMSF, 50 μl of autoclaved Tween 20 and 50 μl of IGEPAL were aliquot and diluted in sterile deionized water. The volume was adjusted to 40ml of autoclaved sterile water.

A.1.14 1 M DTT

0.1543 g of DTT was weighed and dissolved in 1ml of autoclaved sterile water.

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A.2 Preparation of fresh competent *E.coli* cells

The following procedure was modified from that of Sambrook *et al.* Frozen stock of *E. coli* cells (DH5&) were inoculated in LB broth and then incubated overnight at 37 °C with shaking. The 500 ul fresh overnight inoculum was subcultured into a further 50 ml LB broth and incubated with shaking for approximate 2.50-3 hrs at 37 °C until the cells reached logarithmic phase, optimal density at 550 nm was 0.5. Three milliliters of inoculum was aliquot into Focal tubes, chill on ice for 10 min and centrifuge at 2,000 rpm for 5 min at 4 °C in a pre cooled rotor. The *E.coli* cell pellet was resuspended in 1.5 ml ice-cold steriled solution of 50 mM $CaCl_2$ and 10 mM Tris-HCl (pH 8.0) and stand on ice bath for 15 min. This suspension was centrifuged for 5 min at 2,000 rpm in a pre cooled rotor. The *E.coli* cell pellets were then resuspended in 200 µl of ice-cold steriled solution of 50 mM $CaCl_2$ and 10 mM Tris-HCl (pH 8.0), then the cells were transferred to 1.5 ml eppendorf tube.

A.3 Transformation of competent *E.coli* cells

The recombinant molecules of pTaq plasmid (2 µl) were mixed with 200 µl of competent *E.coli* cells. The mixture was incubated on ice bath for 30 min, then heated at 42 °C for exactly 2 min and let stand on ice again for 1 min. One mililiter of SOC/LB medium (with 100 mg /ml Ampicilin) was added to the mixture and incubate for 1 hr at 37 °C. After incubation, the cells suspension was centrifugue at 5,000 rpm for 1 min. The supernatant was discard about 900 µl and then vortex and gently resuspended with pipette tips to disperse cells. The cells then were spreaded on LB agar plate with ampicillin (100 mg/l) and incubated at 37 °C overnight.

3.3 Gene Expression

A single colony (or 3-5 colonies) is used to innoculate 2 ml LB broth, with ampicillin (100 mg/l) and then incubated overnight at 37 °C with shaking. The 100 µl fresh overnight inoculum was subculture into a further 10 ml LB broth with ampicillin (100 mg/l) in a 125 or 250 ml flask. The incubation of the culture is made at 37 °C until the OD_{550} is 0.2 (usually 2-3 hrs) with shaking. The culture is cooled in on ice bath to prevent overgrowth until use. 1.25 mg of IPTG was dissolve to 1 ml LB broth, and then take to 10ml of inoculum and incubate it at 37 °C for 12 hrs (should be

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exactly). The inoculum was transferd to Folcol tube and chill on ice bath. This inoculum was centrifuqed for 5 min at 2,000 rpm, 4 °C in a pre–cooled rotor. The *E.coli* cells pellet were resuspended and vortex gently in 5 ml buffer A. The cell suspesion were centrifuqed for 5 min at 2,000 rpm, 4 °C. The supernatant were discard and the pellet cells were resuspended in 0.5 ml of prelysis buffer (0.5ml buffer A + 2 mg lysozyme). The cell suspension was mixed gently and place at room temperature for 15 min. 5ml of lysis buffer were added and mixed gently. The mixture were transferred into glass tube and incubated at 75 °C for 1 hr. The mixture was transferred into 1.5 ml eppendrof tubes and place on ice bath for 5 min then centrifuge at 1,200 rpm at 4 °C for 10 min. The supernatant were transferred into new 1.5 ml eppendorf tubes. 100 µl of 10 mM DTT was added in a 1 ml of the lysate. The Taq DNA polymerase was assay activity by PCR.

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B. LDLR database

[Accession Number: AY 324609]

[Available from: http://droog.gs.washington.edu/parc/data/ldlr/ldlr.ColorFasta.html]

```
GCTTTAAGTT GAATCTTTAA ACTTATCTTT ATTTTTGAGA CACAGTCTCA 50 | REPEAT
CTCTGTCGCC CAGGCTGGAG TGCAGTGGTA CAACCACAGC TCAGTGCAGC 100
GTTGACCTCC TGGGCTCAAG CCATCCTCCC GCCTCAGCCT CCCGAGTAGC 150
TGGGACTACA GGCGCACACA ACCATGTCCA GCTTATTTTT GTATTTTTTG 200 | var(163):[T:0.01] | var(172):[A:0.01]
TAGAGACAGG GTCCCACTGT GTTGCCCTGG CTTGTTCTGA ACTCCTAGGC 250
TCAAGTGATC CCCCCGCCTC ACCCTCCCAA AGTGCTGGGA TTACAGGCAT 300
GAGCCACCAC ATCCAGACTT CACTTTTTTG TTTAATGTCG CAAATGGCAT 350
AAGGAATGGG ATTCAATGGG GACACATTTA TAAACGTTGC AGCAGCTCCT 400 | var(385):[T:0.18]
AGAACTTGCC TATCCTTGTA AACTTCTCTA GGTGATTGCT AATTACTTCT 450 | REPEAT
TTTTTTTTTT TTTTTTTTGA GACGGAGTCT CACTCTGTCG CCCAGGCTGG 500
AGTACAGTGG CGCAATCTCG TCTCACTGCA AACTCCACCT CCCGGGTTCA 550
CGCCATTCTC CTGCCTCAGC CTCCCGAGTA GCTGGGACTA CAGGCACCCG 600
CCACCACGCC CGGCTAATTT TTTGTATTTT TTTTTAGTAG AGGTGGGGTT 650
TCACTGTGTT ATCCAGGATG GTCTTGATCT CCTGACCTCG TGATCCACCT 700 | var(689):[T:0.01] | var(700):[C:0.20]
GCCTCAGCCT CCCAAAGTGC TGGGATTACA GGCGTGAGCC ACCATGCCCA 750
GCCCGCTAAT TATTTCAATT TGACCTTGAC ACTGAGCCTG CCAAGTAGGT 800
TCAAGCATTT TGATGGCCCC TTTACAGGTT GGGAAAGCTA ATTTATCTGT 850
CCAAGGCCGA ATTCTGAAAC TGAGTCTTAA CTGCCAAAAA TTCTTATCAT 900 | var(881):[A:0.07]
CAATTTCTTC TTCTGGGTTG GGCACAGTGG CTCATGCCTG TAAAGCCAGC 950 | REPEAT
AATTTGAGAG GCATCATGAT GCAAGAGGAA GAGGATTGAG TGAAGCTAGG 1000 | var(953):[G:0.02] | var(978):[A:0.07]
AGTTTGGGAC CAGCCTGGGC AACATAGTGA GACCCCATCT ATAAAAAAAA 1050
ATTAAAAATT AGTTGGGCAT GGTGGTGCAC TCCTGTGGTC CTAGCTATTC 1100
AGGAGGCTGA GGTGGGAGGA TTCCTTGAGC CCAGGGTTGA CGCTGCAGAG 1150 | var(1141):[T:0.01]
AGCTGTGATC ACGCCACTGC AGTCCAGCCT GAGTGACAGC TGGAAATAAT 1200
GATAAATAAA TAATAAATAA TTATTTAAAA AATTATAATA AAAATAATTA 1250
AAAAATTATT TTCCCTGATT AATCTTTTTT TTTGTCCTTC TGAGAGTTCA 1300
ATTTGTCCCT TTTCTGCCTG GTCTCCTAGG TTTCCCTAAA ATCCTGCTGA 1350
GAGGTTAGCA CTGCCTGCCA AAGTCAGTTT GCAAAATCCC AGAGAAATCC 1400
AGCTTATTCC TGGGGGAACC GCCAAGACTG CCCAGCCCTG TGTGGGGTTC 1450

AGGCAAGTTT CTCACATGTG CCTTTTTGGC AAGAGGCCTC TGGCAACCCC 1500 | var(1497):[T:0.01]
ATGAGTCCCC AAAGAGACTC AATTCTAAAA GTTGGTCTCC ACCAGGTCTC 1550
TGTGGCTTAG GGGTTCAAGT TCAACTGTGA AAGCCCTGTT TTGTTTTGAT 1600
TTTGCTTTGA GGGAGAGGAA ACCGCCCTTC TGTTTGTTCA ACTCCTTCTC 1650
CTAAGGGGAG AAATCAATAT TTACGTCCAG ACTCCAGGTA TCCGTACAAT 1700 | var(1694):[A:0.01]
TGATTTTTCA GATGTTTATA CTCAGCCAAA GGCGGGATCC CACAAAACAA 1750 | var(1733):[T:0.01]
AAAATATTTT TTTGGCTGTA CTTTTGTGAA GATTTTATTT AAATTCCTGA 1800
TTGATCAGTG TCTATTAGGT GATTTGGAAT AACAATGTAA AAACAATATA 1850
CAACGAAAGG AAGCTAAAAA TCTATACACA ATTCCTAGAA AGGAAAAGGC 1900
AAATATAGAA AGTGGCGGAA GTTCCCAACA TTTTTAGTGT TTTCCTTTTG 1950
AGGCAGAGAG GACAATGGCA TTAGGCTATT GGAGGATCTT GAAAGGCTGT 2000
TGTTATCCTT CTGTGGACAA CAACAGCAAA ATGTTAACAG TTAAACATCG 2050
AGAAATTTCA GGAGGATCTT TCAGAAGATG CGTTTCCAAT TTTGAGGGGG 2100
CGTCAGCTCT TCACCGGAGA CCCAAATACA ACAAATCAAG TCGCCTGCCC 2150
TGGCGACACT TTCGAAGGAC TGGAGTGGGA ATCAGAGCTT CACGGGTTAA 2200 | var(2155):[T:0.01]
AAAGCCGATG TCACATCGGC CGTTCGAAAC TCCTCCTCTT GCAGTGAGGT 2250 | var(2206):[T:0.01]
GAAGACATTT GAAAATCACC CCACTGCAAA CTCCTCCCCC TGCTAGAAAC 2300
CTCACATTGA AATGCTGTAA ATGACGTGGG CCCCGAGTGC AATCGCGGGA 2350 | Exon 1 | UTR
AGCCAGGGTT TCCAGCTAGG ACACAGCAGG TCGTGATCCG GGTCGGGACA 2400
CTGCCTGGCA GAGGCTGCGA GCATGGGGCC CTGGGGCTGG AAATTGCGCT 2450
                    M  G  P    W  G  W    K  L  R   9
GGACCGTCGC CTTGCTCCTC GCCGCGGCGG GGACTGCAGG TAAGGCTTGC 2500
W  T  V  A    L  L  L    A  A  A    G  T  A              22
TCCAGGCGCC AGAATAGGTT GAGAGGGAGC CCCCGGGGGG CCCTTGGGAA 2550 | var(2507):[A:0.01]
TTTATTTTTT TGGGTACAAA TAATCACTCC ATCCCTGGGA GACTTGTGGG 2600
GTAATGGCAC GGGGTCCTTC CCAAACGGCT GGAGGGGGCG CTGGAGGGGG 2650
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AGGATGGTCT CAATCTCCTG ACCTCGTGAT CCGCCAGCTT TGGCCTCCCA 14600
AAGTGTTGGG ATTACAGGCG TGAGCCACTG AGCCCGCCCC ATATGTATGT 14650
ATATATATAT TTTTTTAAAA TGGGAGACCA GGCATGGTGG CTCATGCCTA 14700 | REPEAT
GAATCCCAGC ACTTTGGGAA GCTGAGGTAG GCGGATCACT TGAGGCCATG 14750
AGTTTGAGAC CAGCCTGCTC AACATGATGA AACTTCTATC TCTACTAAAA 14800
AAAAAAGTGG GATTAGGTCA GGCACGGTGG CTCACACCTG TAATCCCAGC 14850 | REPEAT
ACTTTCAGAG GCCGAGGCAG GAGGATCATG AGGTCAGGAG ATCGAGACCA 14900
TCCTGGCTAA CACGGTGAAA CCCCGTCTCT ACTAAAAAAA TACAAAAAAT 14950
TAGCCAGGCG TGGTGGCGGG TGCCTGTAGT CCCAGCTACT CAGGAGGCTG 15000
AGGCAGGAGA ATGGCGTGAA CCCGGGAGGC GGAGCTTGCA GTGAGCCAAG 15050 | NOT SCANNED
ATCGTGCCAC TGTACTCCAG CCTGGGCGAC AGAGCAAGAC TCTGTCTCAA 15100
AAAAAAAAAA AAAAGTGGGA TTGACATTCT CTTCAAAGTT CTGGGGTTTT 15150
CTTTGCAAAG ACAGGATTGG CAAGGCCAGT GGGTCTTTTT TGTGTGTGTG 15200 | REPEAT
TGTGTGACGG AGTCTCACTC TGCCACCCAG GCTGGAGTGC AATGGCAGGA 15250 | var(15234):[A:0.01]
TCTCGGCTCA CCGCAACCTC CTCCTCCCAG GTTAAAGTGA TTCTCCTGCC 15300
TCAGCCTCCC GAGTAGCTGG GACTACAGGT GCCCGCCACC ACACCCAACT 15350
AATTTTTGTA TTTTTAGTAG AGACAGGGTT TCACTATATT GGCCAGGCTG 15400 | var(15368):[C:0.01]
GTCTTGAACC CCTGACCTCA CGTGATCCAC CCGCCTTGGC CTCCCAAAGT 15450
GCTGGGATTA CAGGCGTGAG CCACTGTGCT CGGCCTCAGT GGGTCTTTCC 15500
TTTGAGTGAC AGTTCAATCC TGTCTCTTCT GTAGTGTCTG TCACCTGCAA 15550 | Exon 3
                                      L  S   V  T  C  K 69
ATCCGGGGAC TTCAGCTGTG GGGGCCGTGT CAACCGCTGC ATTCCTCAGT 15600
  S  G  D   F  S  C   G  G  R  V   N  R  C   I  P  Q   85
TCTGGAGGTG CGATGGCCAA GTGGACTGCG ACAACGGCTC AGACGAGCAA 15650
F  W  R  C   D  G  Q   V  D  C   D  N  G  S   D  E  Q  102
GGCTGTCGTA AGTGTGGCCC TGCCTTTGCT ATTGAGCCTA TCTGAGTCCT 15700
 G  C                                                  104
GGGGAGTGGT CTGACTTTGT CTCTACGGGG TCCTGCTCGA GCTGCAAGGC 15750
AGCTGCCCCG AACTGGGCTC CATCTCTTGG GGGCTCATAC CAAGCCTCTT 15800
CCGCCCTTCA AATCCCCCCT TGACCAGGAG GCATTACAAA GTGGGGATGG 15850
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TGCTACCTCT TCGGGTTTGT CACGCACAGT CAGGGAGGCT GTCCCTGCCG 15900 | var(15864):[A:0.02]
AGGGCTAGCC ACCTGGCACA CACACTGGCA AGCCGCTGTG ATTCCCGCTG 15950
GTCGTGATCC CCGTGATCCT GTGATCCCCG CCCCGTGAGG CTGAACACAT 16000 | var(15954):[A:0.01]
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AAAGCCAGAA AGTTCTGCCA AGGAGCAAGG CCAAGAATCC CGAAGGGAAA 16100
TGGACTTTGA AGCTGGGCGT CTTCTTGGCT GTCTTAATAC AAGTGGCACA 16150 | REPEAT
TCCAAATCCA AAACCCCGAA ATTCAAAGTC TTGAGCACCC GAAATTCTGA 16200
AACGTCTTGA GCACTGACCT TTAGAAGGAA ATGCTTATTG GAGCATTTTG 16250
GATTTCGGAT TTTTACCACT GAGTGTGGAG TCCTAATTAG GAAAAAAACC 16300 | REPEAT
AGGCTGACCG AACCAAAGGA AAGCAATAAA AGAAGGCAGA TAGGGTCAGG 16350 | REPEAT
CACGGTGGCT CACCCCTGTA ATCCCAGCCT TTTGAGAGGC TGAGGCGGGT 16400 | var(16375):[T:0.01]
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CTGTAATCCC AGCTACTCGG GAGGCTGAGA CAGGAGAATC ACTTGAACCT 16550
GGGAGGCAGA GGTTGCAGTG AGCCAATATC ACGCCATTGC ACTCCAGCCT 16600
GGGGGACAAG AGCGAAATTC TGTCTCAAAA AAAAAGAAGA AGAAGGCCGA 16650 | REPEAT
CAAACTATGT AACTCTGCCT TTCTCCATGG TCCAGAACAC ACAGCCCTCC 16700
TGCGTAAATA ACTCCTTATC TTCCTGCTCC CAGCTATCAT CAGACACCTC 16750 | var(16728):[G:0.41]
GGCTGATAGA AAATTGCAAG TTAGCTCACT GCAACCTCGG CATTATAAGT 16800 | var(16780):[C:0.01]
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CTCCAGCAAG CGGCCAGGTG CAGTGGCTCA TACCTGTAAT CCCAGCATTT 16900 | REPEAT
TGGGAGACTG AGGCGGGCGG ATCACCTGAG GTCAGGAGTT TGAGACCAGC 16950
CTGGCCAACA TGGTGAAACC CCGTCTCTAT TAAAAATACA AAAAAATTAG 17000
CCAGGCGTGG TGGCAGGTGC CTGTAATCCC AGCTACTTGG AAGGCTGAGG 17050
CAGGAGAATC GCTTGAACCC GGGAGGTGGA AGTTGCAGTG AGCCGAGATC 17100 | var(17070):[T:0.01]
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AAAAAAAAAA AAATTCCCAG CAAGCCTTTG TCTTCTGGCA GTCAGCTCCT 17200 | NOT SCANNED | REPEAT
CTCTTGCTGA CCTGCTCATT GCTTTCTTGC AAGGTATTTT CCTACCTACT 17250
TTCTGGAATA AATCTGTCTT TCTGTACTTA CAACTACCTT TTTTAAAATT 17300 | REPEAT
TCTTTCTTTT TTGAGATGGA GTCTCACTCT GTTTGCCCAG GCTGGAGTTC 17350
AGTGGTGCAA TCTCAGCTCA CTGCAACCTC TACCTACTGG GTTCAAGCGA 17400
TTCTCCTGCC TCAGCTTCCC GAGTAGCTGG GATTACAGGC GTGCACCAGC 17450
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GCCAAGGTGG TCTTGAACTC CTGACCTCAA GTGATCCTCC CACCTCAGCC 17550 | var(17534):[C:0.01]
TCCCAAAGCG CTAGGATTAC GGCCATGAGC CACTGAGGCC GGCTGCACCT 17600 | var(17590):[T:0.01] | REPEAT
ACAACTGTCT TGATAAATTC TTACCCCCAC ACCACTGGTC CAGATAGTCA 17650

GTGCTCACCC ACAACATTAA GGATATTCCA AATTTGAAAC ATTCCAAAAT 17700 | REPEAT
CAGAAAAATA TTCCAACTCT GAAAATATTC CAAAATCCAA AAAAATTCAA 17750
AATCCAAAAC ACTTCTGGTC CCAAGCATTT TAGAGAAGGG ATACTCAACC 17800
CAAAATAAGG ACAGCAATTC TATAAATTGT GCTACCATCT TGCAGGTCTC 17850 | REPEAT
AGTTTAACAG CTTTACACCT ATTAGCGCAC CAGTGCTCAT AGCAGTGCTG 17900
GGAAATGTGT ACAGATGAGG AAACTGAGGC ACCGAGAGGG CAGTGGTTCA 17950
GAGTCCATGG CCCCTGACTG CTCCCCAGCC CGCCTTTCCA GGGGCCTGGC 18000
CTCACTGCGG CAGCGTCCCC GGCTATAGAA TGGGCTGGTG TTGGGAGACT 18050 | var(18041):[C:0.12]
TCACACGGTG ATGGTGGTCT CGGCCCATCC ATCCCTGCAG CCCCCAAGAC 18100 | Exon 4
                                              P  P  K  T 108
GTGCTCCCAG GACGAGTTTC GCTGCCACGA TGGGAAGTGC ATCTCTCGGC 18150
  C  S  Q   D  E  F   R  C  H  D   G  K  C   I  S  R   124
AGTTCGTCTG TGACTCAGAC CGGGACTGCT TGGACGGCTC AGACGAGGCC 18200
Q  F  V  C   D  S  D   R  D  C   L  D  G  S   D  E  A  141
TCCTGCCCGG TGCTCACCTG TGGTCCCGCC AGCTTCCAGT GCAACAGCTC 18250
 S  C  P   V  L  T  C   G  P  A   S  F  Q   C  N  S  S 158
CACCTGCATC CCCCAGCTGT GGGCCTGCGA CAACGACCCC GACTGCGAAG 18300
  T  C  I   P  Q  L   W  A  C  D   N  D  P   D  C  E   174
ATGGCTCGGA TGAGTGGCCG CAGCGCTGTA GGGGTCTTTA CGTGTTCCAA 18350
D  G  S  D   E  W  P   Q  R  C   R  G  L  Y   V  F  Q  191
GGGGACAGTA GCCCCTGCTC GGCCTTCGAG TTCCACTGCC TAAGTGGCGA 18400
 G  D  S   S  P  C  S   A  F  E   F  H  C   L  S  G  E 208
GTGCATCCAC TCCAGCTGGC GCTGTGATGG TGGCCCCGAC TGCAAGGACA 18450
  C  I  H   S  S  W   R  C  D  G   G  P  D   C  K  D   224
AATCTGACGA GGAAAACTGC GGTATGGGCG GGGCCAGGGT GGGGGCGGGG 18500
K  S  D  E   E  N  C                                   231
CGTCCTATCA CCTGTCCCTG GGCTCCCCCA GGTGTGGGAC ATGCAGTGAT 18550
TTAGGTGCCG AAGTGGATTT CCAACAACAT GCCAAGAAAG TATTCCCATT 18600 | var(18560):[A:0.01]
TCATGTTTGT TTCTTTTTTT TCTTTTCTTT CTTTATTTTG TTTTTGAGAT 18650
GGAGTCTCAC TCTGTGATTT TTTTCATCTC TAAATTTCCT ACATCCATAT 18700
GGCCACCATG AGGCCCCAGG CTGGCCGATG GTTGCTGTTA GCTTATTGGG 18750
AAATCACTGT TTGGAAGGTG CTGGTTGTTT TTTGTTGTTT GTTGTTTTTG 18800 | REPEAT
TTTTTGTTTT TATTTTGAGA CGGAGTCTCG CTCTGTCGCC AGGGTGGAGT 18850 | var(18812):[G:0.44]
GCAGTGGCGC GATCAGCTCA CTGCAACCTC CGCTTCCTGG GTTCAAGCCA 18900 | var(18860):[T:0.01] | var(18895):[G:0.09]
TTCTCCTGCC TCAGCCTCCC AAGTAGCGCG GATTACAGGC ATGTGCCACC 18950
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TAGTCAGGCT GGTCATGAAC TCTTGACCTC AGGTGATCCA CCCGCCTCGG 19050
CCTCCCAAAG TGCTGGGATT ACAGGCGTGC ACTGCTGCAC CCAGCCTTTT 19100 | var(19077):[A:0.02] | REPEAT
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GCACGATTAT GGCTCACTGC GGCCTTGATC TCCTTGGCTC AAGCGATCCT 19200
CTCACTTCAG CCTCTCAAGC AGTTGGAACC ACAGGCTGTA CCACCAAGCC 19250
TGGCCAATTT TTTTGTACAG ACACAGGCTG GTCTTGAACT CCTGGGCTCA 19300
AGCAATCCTC CTGCCTTGGC CTCCCAAAGT GCTGGGATTC CAGGCATGAG 19350
CCGCTGCACC CGGCAAAAGG CCCTGCTTCT TTTTCTCTGG TTGTCTCTTC 19400
TTGAGAAAAT CAACACACTC TGTCCTGTTT TCCAGCTGTG GCCACCTGTC 19450 | Exon 5
                                      A  V   A  T  C   236
GCCCTGACGA ATTCCAGTGC TCTGATGGAA ACTGCATCCA TGGCAGCCGG 19500
R  P  D  E   F  Q  C   S  D  G   N  C  I  H   G  S  R  253
CAGTGTGACC GGGAATATGA CTGCAAGGAC ATGAGCGATG AAGTTGGCTG 19550
 Q  C  D   R  E  Y  D   C  K  D   M  S  D   E  V  G  C 270
CGTTAATGGT GAGCGCTGGC CATCTGGTTT TCCATCCCCC ATTCTCTGTG 19600
  V  N                                                 272
CCTTGCTGCT TGCAAATGAT TTGTGAAGCC AGAGGGCGCT TCCCTGGTCA 19650 | REPEAT
GCTCTGCACC AGCTGTGCGT CTGTGGGCAA GTGACTTGAC TTCTCAGAGC 19700
CTCACTTCCT TTTGTTTTGA GACGGAGTCT CGCTCTGACA CCCAGGCTGG 19750 | REPEAT
AGTGCTGTGG CACAATCACA GCTCACGGCA GCCTCTGCCT CTGATGTCCA 19800
GTGATTCTCC TGCCTCAGCC TCCCGAGTAG CTGAGATTAA AGGCGTATAC 19850
CACCACGCCC GGCTAATTTT TTGTATTTTT ATTAGAGACA GGGTTTCTCC 19900
ATGTTGGCCA GGCTGGTCTT GAACTCCTGG TCTCAGGTGA TCCACCCGCC 19950
TCGGCCTCCC AAAGTGCTAG GATTACAGGT GTGAGCCACT GCGCCAGGCC 20000
TAATTTTTTT GTATTTTTAG TAGAGATGCG GTTTTGCCAT ATTGCCCAGG 20050 | REPEAT
CTGGTCTCGA ACTCCTGGGC TCAAGCGATC TGCCTGCCTT GGCCTCCCAA 20100
AGTGCTGGGA TTACAGGCAC AAACCACCGT GCCCGACGGG TTTTCTTAAT 20150
GAATCCATTT GCATGCGTTC TTATGTGAAT AAACTATTAT ATGAATGAGT 20200
GCCAAGCAAA CTGAGGCTCA GACACACCTG ACCTTCCTCC TTCCTCTCTC 20250
TGGCTCTCAC AGTGACACTC TGCGAGGGAC CCAACAAGTT CAAGTGTCAC 20300 | Exon 6
          V  T  L   C  E  G   P  N  K  F   K  C  H  285
AGCGGCGAAT GCATCACCCT GGACAAAGTC TGCAACATGG CTAGAGACTG 20350
 S  G  E   C  I  T  L   D  K  V   C  N  M   A  R  D  C 302
CCGGGACTGG TCAGATGAAC CCATCAAAGA GTGCGGTGAG TCTCGGTGCA 20400 | var(20394):[T:0.01]
  R  D  W   S  D  E   P  I  K  E   C                    313

GGCGGCTTGC AGAGTTTGTG GGGAGCCAGG AAAGGGACTG AGACATGAGT 20450
GCTGTAGGGT TTTGGGAACT CCACTCTGCC CACCCTGTGC AAAGGGCTCC 20500
TTTTTTCATT TTGAGACAGT CTCGCACGGT CGCCCAGGCT GGAGCGCAAT 20550 | REPEAT
GGCGCGATCT CGGCTCACTG CAACCTCTGC CTCCCAGGTT CAAGTGATTC 20600
TCCTGCCTCA GCCTCCTGAG TAGCTGGGAT TACAGGCGCC CACCACCAAG 20650
CCCGGGTAAT TTTTTGTATG TTTAGTAGAG ATGGGGTTTC ACTATGTTGG 20700
CCAGGCTGGT GTTGAACTCC TGACCTCATG ATCCGCCCAC CTCGGCCTCC 20750
CAAAGTGCTG GGATTACAGG CGTGACCCAC CCCATGAAAA AAAATTAAAA 20800
AATGAAGCGA TGCTGGGCGC GGTGGATCAC GCCTGTAATC CCAGCACTTT 20850 | REPEAT
GGGAAGCTGA GGCAGGCAGA TCACGAGGGC AGGAGATTGA GACCATCCTG 20900
GCTAATACGG TGAAACCCCA TCTCTACTAA AACTACAAAA AATTAGCCGG 20950 | var(20908):[T:0.32]
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AGAATCGCTT GAACCCAGGA GGTGGAGGTT GCAGTGAGCC GGGATCACAC 21050
CATTGCACTC CAGCCTGGGT GACAGAGTGA GACTCTGTCT CAAAAAAAAA 21100
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CCTGCTTCTG TCTTGAGGTT TGTTGTTGTT GTTATTTCGA AACAGAGTCT 21200 | REPEAT | var(21181):[-:0.25]
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GCTGGGATTA CAGCTGAATG CCACCTTGCT GGGCTAATTT TTGTATTTTT 21350
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TACTGAAAGA CCATATTAGG ATAACTGTAC AGTATAGAGA AGGCAGTGGC 21550
AAGTTTTCTC TGTCATATAC CAGAGTGGGC TTGGGCATGG TGGCATACTC 21600 | REPEAT
CTGTAGTCTC AGCTAATCAG GAGGCTGAGG AAGGAGGATC GCTTGGGCCC 21650
AGGAGTTGGA GACTGTAGTG AGCTGTGATC ACACCACCAC ACTTCAATCT 21700
GGGCAACAGA GCAAGAGACC CTATCTCTAA AAAAAAGTAA GTATTTCGGA 21750 | REPEAT
CACTGTGGGC CATACGGTCT CTGGTGCAGT TTCTCAACAT GGCTGTTGGG 21800 | var(21766):[A:0.01]
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GTGTCATGAA AATGATTTCC CTTTTTATTT TTATTTTTCT TCTCAAGTAT 21950
TTAAATATGT AAAAGCCATT TTTAGGCCTG GCAGGATGGT TCACAGCTGT 22000 | REPEAT
AATCCCAGCA CTTTGGGAGG TCGAGGCGGG AGGATCACGA GGTCAGGAGA 22050
TCGAGACCAT CCTGGCCAAC ACAGTGAAAC CCCGTCTCTA CTAAAAATAC 22100
AAAAAATTAA CCAGGCTTGG TGGCGCGCGT CTGTAGTCCC AGCTGCTCAG 22150
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GAGGCTGAGG CAGGAGAATC GCTTGAATGC AGGAGGCGGA GGTTGTAGTG 22200
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GCCTCAAACA AAAAAATGTT TGCCCATGCT GGTCTTGAAC TCCTGGGCTC 22300 | REPEAT
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CTTGTTTTAT GTATATATAT AACTTGTTTT ATATATATAC ATAAACTGCA 22450 | REPEAT
GTAAAAAACA TGTAACATAA AATTTACCTT CTCAAACCTT ATTAAGTGCA 22500
CAGTTCTGTG CCATTAGCAA ATTCACACTG TTGTACAACA TCACAACCAC 22550
CATCTCCAGA ACTTTTTTTT TTTTTTTTAT TCTTTTTGAG ACAGAGTCTC 22600 | REPEAT
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CTCCACCTAC CAGGTTCAAG CAATTCTCCT GCCTCAGCCC CCTCAGTAGC 22700
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CTGACCTCAA GTGATCCTCC CACCTCAGCC TCCCAAAGTG CTGGGAATAC 22850
AGGCATGAGC CACTGCGCCC GGCCCCAGAA CTCTTTTATC TTCCCAAACT 22900 | REPEAT
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CCTCCTCTGA GTGGAATCAG ACAGCATTTT CCTTTTTTGA CTGGCTTATT 23050
TCACTGAGCC AAGTGCGGTG GCACACGCCT GTAATCCCAA AACTTTGGGA 23100 | REPEAT
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CAACATGGTG AAACCCCATC TCTAGTAAAA ATACAAAAAA TTAGCCTGTC 23200
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ACAAAAAAAT TAGCTGGGCA TGGTGGCACA TGCCTGTAGT CCCAGCTACT 23400 | REPEAT
TGGGAGGCTG AGGCAGGAGA ATCACTTGAA CCCGGGAGGT GGAGGTTGTA 23450
ATGAGCCAAG GTTGGCGGCG AAGGGATGGG TAGGGGCCCG AGAGTGACCA 23500 | NOT SCANNED
GTCTGCATCC CCTGGCCCTG CGCAGGGACC AACGAATGCT TGGACAACAA 23550 | Exon 7
                        G  T   N  E  C   L  D  N  N 322
CGGCGGCTGT TCCCACGTCT GCAATGACCT TAAGATCGGC TACGAGTGCC 23600
  G  G  C   S  H  V   C  N  D  L   K  I  G   Y  E  C  338
TGTGCCCCGA CGGCTTCCAG CTGGTGGCCC AGCGAAGATG CGAAGGTGAT 23650
L  C  P  D   G  F  Q   L  V  A   Q  R  R  C   E       353
TTCCGGGTGG GACTGAGCCC TGGGCCCCCT CTGCGCTTCC TGACATGGCA 23700
ACCAAACCCC TCATGCCTCA GTTTCCCCAT CTGTTAAGTG TGCTTGAAAG 23750 | REPEAT
CAGTTAGGAG GGTTTCATGA GATTCCACCT GCATGGAAAA CTATCATTGG 23800 | var(23754):[G:0.09]

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CGGGTGCGTA ATCCCTGTAA TCCCAACACC TTGGGACGCC GAGGCGGGCA 23900
GATCACCTGA GGTCGGGAGT TCCAGACCAG CCTGACCAAC ATGGAGAAAC 23950
CCCGTCTCTA CTAAAAATAC AAAATTAGCC GGGCTTGGTG GTGCATGCCT 24000
ATAATCCCAG CTACTCAGGA GGCTGAGGCA GGAGAATCAC TTGAACCTGG 24050
GAGGTGGAGG TTGTGGTGAG CCAAGATCGT GCCATTGCAC TCCAGCCTGG 24100
GCAACAAGAG TGAAACTCCA TCCAAAAAAA AAAGAAAAGA AAAGAAAAAA 24150
AAGAAAAGAA ATTTCAGCTG ACACAGCTTC ACACTCTTGG TTGGGTTCCC 24200
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CCTTGATTAC ATCTCCCGAG AGGCTGGGCT GTCTCCTGGC TGCCTTCGAA 24300
GGTGTGGGTT TTGGCCTGGG CCCCATCGCT CCGTCTCTAG CCATTGGGGA 24350
AGAGCCTCCC CACCAAGCCT CTTTCTCTCT CTTCCAGATA TCGATGAGTG 24400 | Exon 8
                                  D  I  D  E  C 358
TCAGGATCCC GACACCTGCA GCCAGCTCTG CGTGAACCTG GAGGGTGGCT 24450
  Q  D  P   D  T  C   S  Q  L  C   V  N  L   E  G  G  374
ACAAGTGCCA GTGTGAGGAA GGCTTCCAGC TGGACCCCCA CACGAAGGCC 24500 | NOT SCANNED
Y  K  C  Q   C  E  E   G  F  Q   L  D  P  H   T  K  A 391
TGCAAGGCTG TGGGTGAGCA CGGGAAGGCG GCGGGTGGGG GCGGCCTCAC 24550
 C  K  A  V                                           395
CCCTTGCAGG CAGCAGTGGT GGGGGAGTTT CATCCTCTGA ACTTTGCACA 24600
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GGGAGTCTGC CGCCCTGTTA GGACTTGGGC TTGCCAGGGG GATGCCTGCA 24700
TATGTCCTAG TTTTTGGGAA TATCCAGTTA ACGGAACCCT CAGCCCTACT 24750
GGTGGAACAG GAACCGGCTT TCCTTTCAGG GACAACCTGG GGAGTGACTT 24800
CAAGGGGTTA AAGAAAAAAA ATTAGCTGGG CATGGTGCCA CACACCTGTG 24850 | REPEAT
GTCCCAGCTA CTCAGAAGGC TGAGGCGGGA GGATTGCTTG AGGGCAGGAG 24900 | var(24879):[A:0.08]
GATTGGTTGA TCCTCCCACC TCAGCCTCCG GAGTAGCTGG GACCTCAGGT 24950 | REPEAT
GCATGCCACT ATGCCTGGCT AATTTTCTTT TTTCTTTTTT TTTTTTTTTC 25000 | REPEAT
GAGACGGAGT CTCGCTCTGT TGCCCAGGCT GGAGTGCAGT GGCAGGATCT 25050
CGGCTCACTG CAAGCTCCGC CTCCCGGGTT CACGCCATTC TCCTGCCTCA 25100
GCCTCCCCAG TAGCTGGGAC TACAGGAGCC CGCCACTGCA CCAGGCCAAT 25150
TTTTTTGTAT TTTTAGTAGA GACGGGGTTT CACTGTGTTA GCCAGGATGG 25200 | var(25184):[C:0.04] | var(25185):[A:0.04]
TCTCGATCTC CTGACTTCGT GATCCGCCCA CCTCGGCCTT CCAAAGTGCT 25250
CGGATTACAG GCGTGAGCCA CTGCGCCCAG CCGCTAATTT TCATATTTTT 25300 | REPEAT
AGTAAAAACA GGGTTTCACC ATGTTGGCCA GGCTAGTCTT GAACTCCTGA 25350
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ACCACACCTG GCTATTATTA TTTTTTAGAG ACAGGGTGCT GCTCTATCTT 25450 | REPEAT
CCAGCCTGTA GTGCAGTGCA GCCTCCATCA TAGCTCGCTG CAGCCTTGAC 25500
CTCCTGGGTT CACGTGATCG TCCCGCCTAA GCCTCTGGAG GAGCTGGGAG 25550
TACTGGCATG TGCCACCATG CCTGGTTAAT TTTTTTTTTT TTTTTTTTGA 25600 | REPEAT
GACAGAGTCT CATTCTGTCA CCCAGGCTGG AGTGCGGTGG TGCGATCTTG 25650 | NOT SCANNED
GCTTACTGAA ACCTCCACCT CCCAGGTTCC AGCAATTCTC CTGCCTCACC 25700
CTTCTGAGTA GCTGGGATTA CAGGTTCCGG CTACCAAACC TGGCTAGTTT 25750
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GATTCTCCCG CCTCAGCCTC CCAAAGTGCT GGGATTACAG GCTTGAGCCA 25850
CCGTGCCTGG CTTTTTTTTT TTTTTTTTTT TTTGTGGCAA TAAGGTCTCA 25900 | REPEAT
TTGTCTTGCC CAGGCTAGCC TTATGCTCCT AGCCTCAAGT GATCCTCCTC 25950
CCTCAGCCTC CCAAAGTGCT GGGATTACAG GTGGGCGCCA CTGTGCCTGT 26000
TCCCGTTGGG AGGTCTTTTC CACCCTCTTT TTCTGGGTGC CTCCTCTGGC 26050
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TCCATCGACG GGTCCCCTCT GACCCCCTGA CCTCGCTCCC CGGACCCCCA 26150
GGCTCCATCG CCTACCTCTT CTTCACCAAC CGGCACGAGG TCAGGAAGAT 26200 | Exon 9
 G  S  I   A  Y  L  F   F  T  N   R  H  E   V  R  K  M 412
GACGCTGGAC CGGAGCGAGT ACACCAGCCT CATCCCCAAC CTGAGGAACG 26250
  T  L  D   R  S  E   Y  T  S  L   I  P  N   L  R  N   428
TGGTCGCTCT GGACACGGAG GTGGCCAGCA ATAGAATCTA CTGGTCTGAC 26300
V  V  A  L   D  T  E   V  A  S   N  R  I  Y   W  S  D  445
CTGTCCCAGA GAATGATCTG CAGGTGAGCG TCGCCCCTGC CTGCAGCCTT 26350
 L  S  Q   R  M  I  C   S                              453
GGCCCGCAGG TGAGATGAGG GCTCCTGGCG CTGATGCCCT TCTCTCCTCC 26400 | var(26355):[T:0.09] | var(26379):[T:0.32]
TGCCTCAGCA CCCAGCTTGA CAGAGCCCAC GGCGTCTCTT CCTATGACAC 26450 | Exon 10
         T  Q  L  D   R  A  H   G  V  S   S  Y  D  T 467
CGTCATCAGC AGGGACATCC AGGCCCCGA CGGGCTGGCT GTGGACTGGA 26500 | var(26463):[A:0.29]
  V  I  S   R  D  I   Q  A  P  D   G  L  A   V  D  W   483
TCCACAGCAA CATCTACTGG ACCGACTCTG TCCTGGGCAC TGTCTCTGTT 26550
I  H  S  N   I  Y  W   T  D  S   V  L  G  T   V  S  V  500
GCGGATACCA AGGGCGTGAA GAGGAAAACG TTATTCAGGG AGAACGGCTC 26600
 A  D  T   K  G  V  K   R  K  T   L  F  R   E  N  G  S 517
CAAGCCAAGG GCCATCGTGG TGGATCCTGT TCATGGGTGC GTATCCAGGA 26650
  K  P  R   A  I  V   V  D  P  V   H  G                529
CGCTGAGGGC TGCAGAGGGA ATGGAGGGAG CAGGAAGGAG CTTCAGGAAC 26700 | var(26689):[G:0.41]

TGGTTAGTGG GCTGGGCATG GTGGCTCAAA GCACCTGTAA TCCCAGCACT 26750 | REPEAT
TTGGGAGGCC AAGGTGGGTG GATCATCAAG ACCAGCCTGA CCAACATGGT 26800 | var(26790):[G:0.04]
GAAACCTCGT CTCTACTAAA AATACAAAAA TTAGCCGGGT GTGGTGGTGG 26850
GCACCTGTAA TCCCAGCTGC TCGGGAGGCT GAGGCAGGAG AATCACTTGA 26900 | var(26870):[T:0.03]
ACCTGGGAGA TGGAGGTTGC AGTGAGCCAA GACAGCCCCA CTGCACTCCA 26950
GCCTGGGTGA CAGAGTGAGA CTCCGTCTCA AAAAAAAAAA AAAAAACTAA 27000
ACAAAAAACT GGTTAGTGGC TAGACAACAG GATGGTATCT TCCAAGCCCA 27050
TGGCTGACTC AGCAGCTCCT GGGTCAAGAC ACTGTGACCT GTGTCCCCTG 27100
GCAGGAAGCA TCGCCCCTGC CACCTGCCCG GTGTACTCTG TACCTGTCAG 27150 | var(27130):[A:0.09]
GTGACATCTG CTACCTAAGC ACGTGAGAGG TGGCATTTCA CAGTTTCAGT 27200
GTGGTGCTGA CAACCCGGGA CGCACACTGT CCTTGCAGCT ACAATCAGGA 27250
GGTGAATGTT GGGTTTCCAG CAGAGAACAC TGGAGAAGGC ACACTTGGTG 27300
TCTGGAAGGG AAAAGCAGGG AAGAGAGCAT CATCAGATGC CTGCGGGTGA 27350
AGGTGGGCCC GCTATGGCCA GCGTCCCTTT TTATTTTTAT TTATTTATTT 27400 | var(27352):[A:0.10] | REPEAT
ATTTGAGATG GAATCTCGCT CTGTCGCCCA GACTGTAGTG CAGTGGTGCG 27450
ATCACGGCTC ACTGCAAGCT CCGCCTCACA GGTTCACGCC ATTCTCCTGC 27500
CTCAGCCTCC CGAGTAGCTG GGACTACAGG CACCCGCCAC CACGCCCGGT 27550 | var(27543):[G:0.07]
TAATTTTTTG CATTTTTATT AGAGACGGGG TTTCACCGCG TTAGCCAGGA 27600 | var(27590):[A:0.10]
TGGTCTAAAT CTCCTGACCC TGTGATCCAC CCGCCTCGGC CTCCCTAAGT 27650
GCTTGGATTA CAAGCGTGAG CCACCACGCC CGGCCCCCTT TTTATTTTTT 27700 | REPEAT
ATTTTTTGAG ACGGAGTCTC GCTCTGTCGC CCAGGCTAGA TTGCAGTGGC 27750 | var(27713):[T:0.30] | var(27729):[A:0.01] | var(27750):[T:0.02]
GTGATCTCGG CTCACTGCAG CCTCCGCCTC CCAGGTTCAA GTGATTCTCC 27800
TGCCTCAACC TCCCAACTAA TTAGGATTAC AAGCATGTAC CACCATGCCT 27850
GACTAATTTT TTGTATTTTT AGTAGAGACT GGGTTTCACC ATGTTGGCTA 27900
GGCTGGTCTC GAACCCTTAG CCTCAAGTAA TCTGCCTGCC TCAGCCTCCC 27950
AAACAGCGGG GATTACAGGC ATGAGCCACT GTGCCCAACC CAACCCTGGA 28000
TCTCTTTTAA ACAAGACAAT GCTCGCTGTT GCCACAGAAC AATGGGTGGG 28050
GTACATGTGG CCCAGTGTGT TTGGCCACAT AACTGCCAGG CCAGAGGGAA 28100
AGAGACTCTC AGACTGTCTC CACTCAGATA CAAATGTGTG TGTTGTGTGC 28150
GTGTGTTCTG GTCTCATATT TGTTTGTTTT GAGACAGGGT GTCGCTCTGT 28200 | REPEAT
CACTGAGTCT GGAGTGCAGT GGCGCAATCA GAGTTCACTG CAGCCTCAAA 28250
CTCTTGGGCT CAGTTGATTC TCCCACTTCA GCCTCCCAAG TAGCTGGAAC 28300
TACAGGTGAA CACCACTGTG CCCAGCTAAT TTATTTTATT TTTAGTAGAG 28350
ATGAGGTCTC ACTATGTTGC CCAGGCTGGT CTTGACCTCC TAGCCTCAAG 28400
CAATCCTCCT GCCTTGGTCT CCCAAAGTGC TGGGATTACA CGTGCGAGCC 28450 | var(28442):[A:0.02]
ATTGCGCATG GCTTGTGTTC TTGTGTTTCT TCCTTTTTCT TTCGAGATGG 28500 | var(28455):[T:0.01] | REPEAT
CGTCTCAGTC TGCCACCCAG GCTGGAGTGC AGTGGTGTGA TCATAGCTCA 28550 | var(28520):[C:0.02]
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CTGTAGCCTC AACTTCCTGG GCTCAAGCAA TCCTCTTGAT TTCAGCCTCC 28600
CGGGCCTGGC CAGCATGGTG AAACCCCGTC TCTACTAAAA ATACAAAAAT 28650 | REPEAT
GTAGCCAGGC GTGGTGGTGG GCGCCTGTAA TCCCAGCTAC ACCAGAGGCT 28700
GAGGCAGGAG AATCGCTTGA GCCTGGAAGG TGGAGGTTGC AGCAAGCCAA 28750 | var(28730):[T:0.05] | var(28741):[G:0.27] | var(28748):[A:0.01]
GATCGTGCCA CTGCACTCCA GCCTGGGCAA CAGAGACAGA CTCTGTCTCA 28800 | var(28766):[T:0.07]
AAAAAAAAAA AAAAAAACCC AAACAAGCCA CATTTGGAGT TTGGGGTTCC 28850 | var(28817):[C:0.01]
CAGCAGGACT ATTTCCCAAG CCTGAGCCTG GCTGTTTCTT CCAGAATTCG 28900
TTGCACGCAT TGGCTGGGAT CCTCCCCCGC CCTCCAGCCT CACAGCTATT 28950
CTCTGTCCTC CCACCAGCTT CATGTACTGG ACTGACTGGG GAACTCCCGC 29000 | Exon 11 | var(28998):[T:0.08]
                    F   M   Y   W   T   D   W   G   T   P   A 540
CAAGATCAAG AAAGGGGGCC TGAATGGTGT GGACATCTAC TCGCTGGTGA 29050
  K   I   K   K   G   G   L   N   G   V   D   I   Y   S   L   V  556
CTGAAAACAT TCAGTGGCCC AATGGCATCA CCCTAGGTAT GTTCGCAGGA 29100
T   E   N   I   Q   W   P   N   G   I   T   L                  568
CAGCCGTCCC AGCCAGGGCC GGGCACAGGC TGGAGGACAG ACGGGGGTTG 29150 | var(29142):[T:0.26]
CCAGGTGGCT CTGGGACAAG CCCAAGCTGC TCCCTGAAGG TTTCCCTCTT 29200 | REPEAT
TCTTTTCTTT GTTTTTTCTT TTTTTGAGAT GAGGTCTTGG TCTGTCACCC 29250
AGGCTGGAGT GCACTGGTGC AATCGTAGCT CACTGCAGCC TCCACCTCCC 29300 | var(29268):[C:0.43] | var(29295):[T:0.24]
AGGCTCAAGT GATCCTCCTG CCTCACCCTC CTGAGTAGCT GAGATTACAG 29350
ACACGTGCCA CCACGGCAGA CTAATTTTAT TTTATTTTTG GGAAGAGACA 29400
AAGTCTTGTT ATGTTGGCCT GGCTGGTCTC AAACTCAGGG TGCAAGCGAT 29450
CCTCCCGCCT CAGCCTTCCA AACTGCTGGG ATTACAGGCG TGGGCCACCG 29500 | var(29490):[A:0.08]
TACCCAGCCT CCTTGAAGTT TTTCTGACCT GCAACTCCCC TACCTGCCCA 29550 | var(29524):[G:0.29]
TTGGAGAGGG CGTCACAGGG GAGGGGTTCA GGCTCACATG TGGTTGGAGC 29600
TGCCTCTCCA GGTGCTTTTC TGCTAGGTCC CTGGCAGGGG GTCTTCCTGC 29650
CCGGAGCAGC GTGGCCAGGC CCTCAGGCCC CTCTGGGACT GGCATCAGCA 29700 | var(29664):[T:0.25] | var(29678):[A:0.28]
CGTGACCTCT CCTTATCCAC TTGTGTGTCT AGATCTCCTC AGTGGCCGCC 29750 | var(29723):[A:0.01] | Exon 12
                                D   L   L   S   G   R   574
TCTACTGGGT TGACTCCAAA CTTCACTCCA TCTCAAGCAT CGATGTCAAC 29800
L   Y   W   V   D   S   K   L   H   S   I   S   S   I   D   V   N  591
GGGGGCAACC GGAAGACCAT CTTGGAGGAT GAAAAGAGGC TGGCCCACCC 29850
 G   G   N   R   K   T   I   L   E   D   E   K   R   L   A   H   P 608
CTTCTCCTTG GCCGTCTTTG AGGTGTGGCT TACGTACGAG ATGCAAGCAC 29900
  F   S   L   A   V   F   E                              615
TTAGGTGGCG GATAGACACA GACTATAGAT CACTCAAGCC AAGATGAACG 29950
CAGAAAACTG GTTGTGACTA GGAGGAGGTC TTAGACCTGA GTTATTTCTA 30000

TTTTCTTCTT TCTTTTTTTT TTTTTTTTTG AGACAGAGTT TTGCTCTCGT 30050 | REPEAT
TTCCCAGGCT GGAGGGCAAT GGCATGATCT CGGCTCACCG CAACCTCCAC 30100
CTCCCAGGTT CAAGTGATTC TCCTGTCTCA GGCTCCCCAG TAGCTGGGAT 30150
TACAGGCATG CACCACCACC ATGCCCGGCT AATTTTGTAT TTTTAGTAGA 30200
GACGGAGTTT CTCCATGTTG GTCAGGCTGG TCTCGAACTC CCGACCTCAG 30250
GTGATCTGCC TGCCTCGGCC TCCCAAAGTG CTGGGATTAC AGACTTGAGC 30300
CACCGCGCCC AGCTATTTGT GTTTTCTTTC TTTCTTCTTC TTCTTTTTTT 30350 | REPEAT | var(30337):[A:0.22]
TTTTCTAAGA GACAGGATCT CACTCTGTCC CCAGGCAGGA GTGCAGTGCT 30400
GTGATCATAG CTCACTGCAG CCTTAACCTC CTGGGCTCAA GTGATCTTCC 30450
CACCTCAGCC TCCCAAGTAG CTGGAACTAC AGGTGCACAC CACCATGCCC 30500
AGCTCATTTT TGTATTTTTT TTTTTTTTGA GACAGTCTCG TTCTGTCACC 30550 | REPEAT | NOT SCANNED
CCGGCTGGAG TGCAGTGGTA CAATCTTGGC TCACTGCAAC CTCTGCCTCC 30600
CAGGTTCAAG CGATTCTCCT GCCTCAGCCT CCTGAGTAGT TGAGATTACA 30650
GGCATGTGTG CCATCATACC TGGCTGATTT TTGTATTTTT TTTTAGAGAT 30700
GGGGTCTCAG TATGTTGACC AGGCTTGTCT TAAACTCCCG GCCTCAAGTG 30750
ATCCTCCCAC TTCAGTCTCC CAAAGTGCTG GGATTACAGG CATGAGCCAC 30800
TGCGGCCGGT TTGTTTTCTT TTTTTTTTCG TTTTTTGGAG ACGGAATTTC 30850 | REPEAT
ACCTTTGTTG CCCAGGATGG AGTGCAATGG CACGATATCG CCTCACCACA 30900
ACCTCTGCCT CCTGGGTTCA AACCATTTTC CTGCCTCAGC CTTCTTAGTA 30950
GCTGGGATTA CAAGCATGTG CCACCACGCC CGGCTGATTT TGTATTTTTA 31000 | var(30981):[T:0.35]
GTAGAGATGG GGTTTCTCCA TGTTGGCCAG GCTGGTCTCG AACTCCTGAC 31050
CTCAGGTCAT TCGCCCACCT CTGCCTCCCA AAGTGCTGGG ATTACAGGCG 31100 | var(31063):[A:0.22]
TGAGCCACCG TGCCCGGTGG TTTGTATTCT TTTTACTGAG AGTCGTGAAA 31150 | var(31110):[A:0.03] | REPEAT
GGCAGTGATC CTCTGTCACA TGTGATCTTG GCTCTCAGGG GACATTTGGC 31200 | var(31151):[A:0.01] | REPEAT | var(31163):[A:0.36]
AATTTCTAGA GATTTTTTGG TTGTCACAAG TCAATGGGGA AGACTGTTGG 31250
CATTTAGTGG GTAGAGGCTG GTGACGCTGC TGAACACCCA GAACAGGGAA 31300
GTAGCAGGCC CTAGATAGAG CCATCGTGGG GAAACCCTGC TCTAAGGAAA 31350
TGGCGCTATT TTATAACCCC ACGTTCCTGG CATGATTACC AACAGCCAAA 31400
AGTGGAGTCC CCCCAAGTGT GTTCGTCCAT TTGCATTGCA GTAAAGGAAT 31450 | var(31416):[G:0.29] | REPEAT | var(31425):[A:0.03]
AGCTGAGGCC GGGTAATTTA TAAAGAAAAG AGATTTAAAC TGGGTATGGC 31500 | REPEAT
AGTTTATGCC TATAATCCCA GAACTTTGGG AGGCTGAGGC AGGAGGATCG 31550
CTTGAGTCCA GGAGTGTGAG ACCGAGACCA GCCTGGCCAA CATGACGAAA 31600
CTCTGTCTCT ACAAAAAATA CAAAAAGTAG GCCAGGCACG GTGGTTCACG 31650 | REPEAT
CCTGTAATCC CAGCACTTTG GGAGGCCGAG GCGGGCGGAT CACGAGGTCA 31700
GGAGATCGAG ACCATCCTGG CTAACACGGT GAAACCCCGT CTCTACTAAA 31750 | var(31722):[C:0.09]
AATACAAAAA CAAAATTAGC CGGGTGTGGT GGCAGGCGCC TGTAGTCCCA 31800 | var(31775):[C:0.36]
GCTACTCGGG AGGCTGAGGC GGGAGAATGG CGTGAACCCG GGAGGCGGAG 31850
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CTTGCAGTGA GCCAAGATCG CGCCACTGCA CTCCAGCCTG GGTGACCGAG 31900
TTGAGACTCC GTCTCAAAAA AAAAAAAAAA AAAAAAAATA CAAAAAGTAG 31950 | NOT SCANNED | REPEAT
CCAGGTGTGG TGGCAGGCAC CTGTAATCCT GGGTTCTCGA GACCGAGGCA 32000
TGAGAATTGC CTGACCCCAG GAGGTGGAGG CTGCAGTGAG CCAAGATCAT 32050
GCCACTGCAC TCCAGCCTGG GCGACAGAGT GGGACTCTGT CTCAAAAAAC 32100
AACAAAAAAA AAGTTCTGGA AATGGATGGT GGTGATGGTG ATACTTCCAC 32150 | REPEAT
AACAGCGTGA ATCTGCTTAA GGCCACCGAA CTGTGCACTC ACAAATAGTC 32200
GAGATGGTAC ATTTTATGTT ATGTGTATTT CACCACAATT AAAAACTAGT 32250
TGTGGGCCAG GTGTGGTGGT TCATGCCTGT AATCCCAGCA CTTTGGGAGG 32300 | REPEAT
TCAGAGGGAG GTGGATCATG AGGTCAGCAG TTCGAGACCA GCCAGGCCAA 32350
CATGGTGAAA CCCCATCTCT ACTAAAAATA CAAAAATTAG CCAGGCGTGG 32400
TGGCACATGC CTGTAGTCCC AGCTACTTGA GAGGCTGAAG CAGGAGAATC 32450
GCTTGAACCT GGGAGGCTAA GATTGCAGTG AGCCGAGATC GTGCCACTGC 32500
ACTCCAGCCT GGACGACAGA GTGAGACTTC GTCTCAAAAA AAAAACCAAA 32550
AAAAAAATTA GCTGTGGGTC AGGCACTGTG GCTCACGCCT GTAATCCCAG 32600 | REPEAT
CACTTTGGGA GACCGAGGTA GGTGGATGGC CTGAGGTCAG GAGTTCGAAT 32650
CCAGCCTGGC CAACATGGTG AAAGCCCGTC TCTACTAAAA ATACAAAAAA 32700
TTAGTCAGGT ATGTTGGCAC ACCTGTAATC CCAGCTACTC GGGAGGCTGA 32750
AGCAAGAGAA TCGTTTGAAC CCAGGAGGTG GACGTTGCAG TGAGCCGAGA 32800
TTGGGCCACT GTACTCCAGC CTGGGCAACA AAAGTGAAAC TCTGTCTGAA 32850
ACAAACAAAC AAACAAACAA ACAGACAAAC AAAAAAACTA GTTGTGGAGA 32900
GAGGGTGGCC TGTGTCTCAT CCCAGTGTTT AACGGGATTT GTCATCTTCC 32950
TTGCTGCCTG TTTAGGACAA AGTATTTTGG ACAGATATCA TCAACGAAGC 33000 | Exon 13
                D  K   V  F  W   T  D  I   I  N  E  A 627
CATTTTCAGT GCCAACCGCC TCACAGGTTC CGATGTCAAC TTGTTGGCTG 33050
  I  F  S   A  N  R   L  T  G  S   D  V  N   L  L  A   643
AAAACCTACT GTCCCCAGAG GATATGGTTC TCTTCCACAA CCTCACCCAG 33100 | var(33079):[C:0.37]
E  N  L  L   S  P  E   D  M  V   L  F  H  N   L  T  Q  660
CCAAGAGGTA AGGGTGGGTC AGCCCCACCC CCCCAACCTT GAAACCTCCT 33150
 P  R                                                  662
TGTGGAAACT CTGGAATGTT CTGGAAATTT CTGGAATCTT CTGGTATAGC 33200
TGATGATCTC GTTCCTGCCC TGACTCCGCT TCTTCTGCCC CAGGAGTGAA 33250 | Exon 14
                                          G  V  N  665
CTGGTGTGAG AGGACCACCC TGAGCAATGG CGGCTGCCAG TATCTGTGCC 33300
  W  C  E   R  T  T   L  S  N  G   G  C  Q   Y  L  C   681
TCCCTGCCCC GCAGATCAAC CCCCACTCGC CCAAGTTTAC CTGCGCCTGC 33350
L  P  A  P   Q  I  N   P  H  S   P  K  F  T   C  A  C  698

CCGGACGGCA TGCTGCTGGC CAGGGACATG AGGAGCTGCC TCACAGGTGT 33400
 P  D  G   M  L  L  A   R  D  M   R  S  C   L  T       713
GGCACACGCC TTGTTTCTGC GTCCTGTGTC CTCCAACTGC CCCCTCCTGA 33450 | NOT SCANNED | REPEAT
GCCTCTCTCT GCTCATCTGT CAAATGGGTA CCTCAAGGTC GTTGTAAGGA 33500
CTCATGAGTC GGGATAACCA TACTTTTCTT GGATGGACAC ATCAGCACCG 33550
GGCTTGACAT TTACCCAGTT CCCCTTTGAT GCCTGGTTTC CTCTTTCCCG 33600
GCCCCCTGAA GAGGTGATCT GATTTCTGAC AGGAGCCCTG AGGGAGGAAA 33650
TGGTCCCCTT TGTTGACTTT TCTTTTTCTT TATTTTTTTC TTTTGAGATT 33700 | REPEAT
TGCTGTCACC CAGCCTGGAA TGCAGTGGTG CCATCTTGGC TCACTGCTAC 33750
CTCTCCCACT GGGTTCAAGC AATTCTCCTG CCTCAGCCTC CCAAGTAGCT 33800
GGGATTACAA GCATGCGCCA CCATGCCTGG CTAAGTTTTG TATTTTTAGT 33850
ACAGACAGGG TTTCTCCATG GTGGCCAGGC TGGTCTTGAA CTCCTGACCT 33900
CAGGTGATCC TCCCACCTCT GCCTCCCGAA GTGCTACGAT TACAGGCATG 33950
AGCCACCGCG CCCATCCCCC TTTGTTGACT TTTCTCATCC TCTGAGAAAG 34000
TCTCAGTTGA GGCCAGCACC TCCCTCAAGT GAATTGAATC TCCCTTTTGA 34050
ACAACAACAA ATAACAATAT GACCCAGACG TGGTGGCTCA CACCTGTGGT 34100 | REPEAT
CCCAGCTACT CGGGAGGCTG AGGTGTGAGG ATTGCTTGAG CCCAGGAGGT 34150
CAAGGCTACA GAGAGCTATA ATCACACCAC TTCACTCCAG CCTGGGGGAC 34200
AAAGTGAAAC CCTGTCTGAA AAAAACAAAA AAAGAAAAAG GAAAAAGAAA 34250
CAATACGATC ACAAAGTAGA TATTCATAGT GTTTATTTTC AGTACTCTTT 34300 | REPEAT
TTTTTTTTTT TTTTTTTTTT TGAGACGGAG TCTTGCTCTG TTGCCCAGGC 34350 | NOT SCANNED
TGGAGTGCAG TGGCACGATC TTGGCTCACT GCAGCCTCTG CCTCCCAGGT 34400
TCAAGCGCTT GGCTCACTGC AACCTCCGCC TCCTGGGTTC AAGCGCTTCT 34450
TCTGCCTCAG CCTCCCCAGT AGCTGGGACT ATAGGCACGT CCCACTACGC 34500
CCAGCTAATT TTTTGTATTT TTTAGTAGAG ATGGGGTTTC ACTATGTTAG 34550
CCAGGATGGT CTCGATCTCC TGACCTCGTG ATCTGCCTGC CTTGGGCTCC 34600
CAAAGTGTTG GGATTATGGG CATGAGCCAC TGCACCTGGC CTTTTTTTTT 34650 | REPEAT
TTTTTTTTTG AGATGGAGTT TCGCTCTTGT TGCCCAGGCT GGAGTGCAAT 34700
GGTGTGATCT CGGCTCACTG CAACCTCTGC CTCCTGGGTT CAAGCAATTC 34750
TCCTGCCTCA GCCTCCCGAG TAGCTGGGAT TACAGGCACC TGCCACCACG 34800
CCTGGCTAAT TTTTGTACTT TTAGTAGAGA CGGGGTTTCT CCATGTTGGT 34850
CAGGCTGGTC TCAAACTCCT GACCTCAGGT GATCCACCCA CCTCGGCCTC 34900
CCAAAGTTCT GGGATTACAG ACATGAGCCA CCGCGCCTGG CCGTGTCTGG 34950
CCTTTTTTAG TTATTTCTTT TTTTTTTTTT TTTTTTTTTG AGACAGAGTC 35000 | REPEAT
TTACTCCGTC GCCCAGGCTG GAGTGCAGCG GTGCGATGTC TGCGCACTGC 35050
AAGCTCCGCC CCCTGGGTTC ATGCCATTCT CCTGCCTCAG CCTTCTGAGT 35100
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AGCTGGGACT GCAGGCGCCT GCCACTACGC CCGGCTACTT TTTTGTATAT 35150
TTAGTAGAGA TGGAGTTTCA CTGTGTTAGC CAGGATGGTC TCGATCTCCT 35200
GACTTTGTGA TCCGCCCGCC TCGGCCTCCC AAAGTGCTGG GATTACAGGC 35250
GTGAGCCACC ATGCCAGGCT TTTTTTTTTT TTTTTTTTTT TGAGACGGAG 35300 | REPEAT
TCTTGCTCTG TCGCCCAGGC TGGAGTGCAG TGCCATGATC TCAGCTCACT 35350
GCAAGCTCCA CTTCCCAGGC TCACGCCATT CTCCAGCCTC AGCCTCCCAA 35400
GTAGCTGAGA CTACAGGGGC CCGCCACCAC ACTCGGCTAA TTTTTTTGTA 35450
TTTTTAGTAG AGACGGGGTT TCACCATGTT AGCCAGGCTG GTCTTGAACT 35500
CCTAACCTCA GGCGATTCAC CTGCCTCGGC CTCCCAAAGT GCTGGGATTA 35550 | NOT SCANNED
AAGGTATGAG CCACCTCGCC TGGTGTGAGC CACCTCGCCC AGCCTGAGCC 35600
ACCTCACCCA GCCTAAGCCA CTGTGCCTGG CCTGATTTTG GACTTTTTAA 35650
AAATTTTATT AATAATTATT TTTGGGTTTC TTTTTTTTGA GACAGGGTCT 35700 | REPEAT
TACTCTGTCA TCCAGGCCAT CCTGTCTGTC TGTCATCCCA GTGATGGGAT 35750
CATACCTTGC TGCAGCCTCT ACCTCCTGGG CTCAAGCGAT CCTCCCCCCT 35800
CAGCCTCCTG AGTAGCTGGG AGTACAGGTG TGCACCACCA CACCTGGCTA 35850
ATTTTTTTTT TTTTTTTTGT ATATAGAGAT GGTATTTTGC CATGTTGACC 35900 | var(35876):[C:0.19]
AGGCTAGTCT TAAACTCCTG GACTCACTCA AGAGATCCTC CTGCCTTGGC 35950
CTCCCAAGGT CATTTGAGAC TTTCGTCATT AGGCGCACAC CTATGAGAAG 36000
GGCCTGCAGG CACGTGGCAC TCAGAAGACG TTTATTTATT CTTTCAGAGG 36050 | Exon 15
                                                E   714
CTGAGGCTGC AGTGGCCACC CAGGAGACAT CCACCGTCAG GCTAAAGGTC 36100
A  E  A  A   V  A  T   Q  E  T   S  T  V  R   L  K  V  731
AGCTCCACAG CCGTAAGGAC ACAGCACACA ACCACCCGGC CTGTTCCCGA 36150 | var(36139):[A:0.18]
 S  S  T   A  V  R  T   Q  H  T   T  T  R   P  V  P  D 748
CACCTCCCGG CTGCCTGGGG CCACCCCTGG GCTCACCACG GTGGAGATAG 36200
  T  S  R   L  P  G   A  T  P  G   L  T  T   V  E  I   764
TGACAATGTC TCACCAAGGT AAAGACTGGG CCCTCCCTAG GCCCCTCTTC 36250
V  T  M  S   H  Q                                      770
ACCCAGAGAC GGGTCCCTTC AGTGGCCACG AACATTTTGG TCACGAGATG 36300
GAGTCCAGGT GTCGTCCTCA CTCCCTTGCT GACCTTCTCT CACTTGGGCC 36350 | var(36308):[C:0.01] | var(36314):[A:0.02]
GTGTGTCTCT GGGCCCTCAG TTTCCCTATC TGTAAAGTGG GTCTAATAAC 36400 | REPEAT
AGTTCTTGCC CTCTTTGCAA GGATTAAATG GGCCAAATCA TATGAGGGGC 36450
CAGGTCCTTC AGGCTCCTGG TTCCCAAAGT CAGCCACGGA CCGTGTGGGT 36500
CCCAAAATTT TATCAAGGCA CATTCGTTGC CTCAGCTTCA GGCATCTGCC 36550 | var(36525):[T:0.03]
CAAAAAGGCC AGGACTAAGG CAAGGAGAGG GAGGGATTCC TCAGTACTCA 36600

GCTTTTCACA GAGGCTCCAA AAGGCTAAGG AATCCAGTAA CGTTTTAACA 36650
CAATTTTACA ATTTTTTTTT TTGAGACGGA GTTTTGCTCT TGTTGCCCAG 36700 | REPEAT
GCTGGAGTGC AGTGGCACGA TCTCGGCTCA CTGCAACCTC TGGCTCCCGG 36750
GTTCAAGCGA TTCTCCTGCC TCAGTCTCCC GAGTAGCTGG GATTACAGGC 36800 | var(36789):[A:0.43]
ATGCGCCACC ACGCTCGGCT AATTTTGTAT TTTTAGTACA GAAGGGGCTT 36850
CTCTGTTGGT CAGGCTGGTC GTGAACTCTC AACCTCAGGT GAGCCACCCG 36900
CCTGAGCCTC CCAAAGTGCT GGGATTACAG GTGTGAGCCA CCACGCCTGG 36950
CCTTTTTTTT GAGACAGAGT CTCGCTCTCG CCCATGCTGT ACTGCAGTGA 37000 | REPEAT
CGCAGTCTGG GCTCACTGTA ACCTCCGCTT CCCAGGTTCA AGTGATTCTT 37050
CTGCCGCAGC CTCCCATGTA GAGTAGCTGG GATTACAGGC ACCCGCCACC 37100
ATGCCTGGCT AATTCTTGCA TTTTTAGTAG AGATGGGGTT TCACAGTGTT 37150
GGCCAGGCTG GTCTCAAACT TCTGACCTCA AGTCATCTGC CTGCCTTGGC 37200
CCTGCCAAAG TGCTGGGATT ATAGATGTGA GCCACCGGCG CTGGCCTACA 37250
GTTTATTCTT TGGTGGCTCA CACCTGTAAT CTCAGCACTT TGGGAGGCCA 37300 | REPEAT | var(37288):[T:0.21]
AGGTGGGAGA ATGGCTTGAG CCCAGGAGTT CAAGTCCAGC CTGGGCAACA 37350
TAGCAAGACC CTATCTCTAC TACAAAATAA ATAATAAATA AACTAATTTT 37400
TTTTCTTTTA AAACCCAACT ATTCAACATG GCAATGCAAT ATATTAAAAA 37450
AATTTTTTTT TTCTTTGAAA CGGAGTCTCT CACTGTCACC CGGGCTGGAG 37500 | REPEAT | var(37453):[A:0.04]
TGCAGTGTCG CCATCTTGGC TCACTGCAAC CTCCGCCTCC CAGGTCCAAG 37550 | var(37535):[A:0.07]
TGATTCTCCT GCTTCAGCCT CCCGAGTAGC TGGGATTACA GGCACCCACC 37600
ACCATACCCA GCTAATATTT TTGTATTTTT AGTAGAGATG GGGTTTCACT 37650
ATGTTGGGCA GGCTGGTCTG GAACTCCTGA CCTCGTGATC TGCCCGAGGA 37700
TCGGCGGCCT CCCAAAGTGC TGGGGATTGC AGGCATGAGC CACCGTGCCC 37750 | var(37702):[T:0.20] | var(37703):[A:0.10]
AGCCAAAACT TTTTTATTTT TATTTTTTTG GGACACGGTC TCACTGTGTA 37800 | REPEAT
CCCCAGACTG GAGTGATAGA GTGCTGTCAT GGCTCACTGC AGCCTCAACC 37850 | var(37828):[A:0.01]
TCCCTGGGCT CAGGTGATCT TCCTGCTTCA GTCTCCCAGG TAGCTGGGAC 37900
TACAGGCATG AGCCACCACA CCCAGCTAAT TTTTGAATTT TTTTGTAGAG 37950
ACAGGGTTTC ACCTTGTGGC CCAGACTTGT CTCTAACTCC AGGGCTCAAG 38000
CGATCTGCCC ACCTTGGCCT CCCAAAGTGC TGAGATTAAT GCAATTTAAA 38050
AAATTTTTTG GCCAGGCCTG GTGGCTCATG CCTGTATTCA CAACACCTTG 38100 | REPEAT
GGAGGCAAAG GTGGGCAGAT CACTTGAGGT CAGGAGTTCG AGACTAGCCT 38150
GGCCAACATG GTGAAACCCC CTGTCTACTA AAAAAATACA AAAATTACCT 38200
GGGCACAGTG GTGGGTGCCT GTAATCCCAG CTACTTGGGA TGCTGAGGGT 38250
GGAGAATTGC TTGAACCTGG GAGGCAGAAG TTGCAGTAAG CCAAGATCAT 38300
GCCACTGGAC TCCAGCCTCA GTGACAGAGC AAAACTCTGT CTCCAAAAAA 38350
ATTGTTTTTT TTTTTTTTTT TTCAAATCAT CACACTACAG CCAAGGCCTG 38400 | var(38378):[A:0.29] | REPEAT
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GCCACTTACT TTTGTAAATA AAGTTTTATT GGAGCCAGTG GACCAGTGAG 38450
GCCGAATCTT GCAGGTGTAA GATCACAGTC TATCCTTGAA AATTTTGATA 38500
TTTTGTTCAT TGGGTGGTTT TTCATTAATT TAAATTTTAA AAAATAACAT 38550
ATTAAAGGCT GGTGTGGAGG TGCACGCCTG CAGTCCTAGC TACTCCCAGA 38600 | REPEAT | var(38576):[A:0.45]
GGCTGAGGCG GGAGACTTGC TTGAGCCCAA GAGTTGAAGT CCAGCCTGGG 38650 | var(38609):[T:0.02]
CAACATAGCG AGACCCCCAT CTCTAAAAAT AAAAATAATG CATTAGAATA 38700
TTATTGGATT CCTGGGCAGG GCACAGTGGC TCACACCTGT AATCCCAGCA 38750 | REPEAT
CTTTGGGAGG CTGAGGTGGG TGGATCACCT GAGGTCAGGA GTTTGAGACC 38800
AGCCTGGCCA ACATGGTGAA ACCCCGTCTC TACTAAAAAT ACAAAAATTA 38850
GCCAGGCGTG GTGGCAGGTG CCTGTAATCC CAGCTACTCG GGAGGCTGAA 38900 | var(38879):[T:0.01]
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CTGCAAAGAA AAGGAATATT ATCAGATTCC TAAGCTTTTT GGCTCCCCCT 39050 | var(39002):[C:0.33]
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CCTGGATGTG ATGAGACCCA GGTGTGGGTC AGGATGTCAT TCGTTTGTCC 39150
ACCAGAGGGC GCCCAAACTG CTTTGAGCTG CTGGGAAATG GTGCTCCTAG 39200
ACTTTTAGCA AACAAACAAA AAAAAATGGC ACATCGGCAA ATTTCAGACC 39250 | var(39203):[C:0.08]
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GTTACAAGCA GGATAAAATG GAAACTGCCT GGGAGAGGCT GAGAAACCTT 39350
CTTGCTTGGG GGAGGTGGGG CACTGCTAGA ATTAATCGCT TCACAGACCA 39400
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TCATTTATGT AGAGACGAGG GGGATCTGGC TATATTGCCT AGATTGGTCT 39500 | REPEAT | var(39467):[A:0.21] | var(39494):[C:0.35]
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GCCTTGCCCA GGGCCCTTAT GTTGAATGGC CCAGGTCCAC TTGTATGGTT 39650
CTGTACCAAG GTTAACCCCA TCCCATAATG CCTGGGACAG TTGATGCAGG 39700 | var(39655):[G:0.01]
ACAATCAGCT TCTGTGCCAT TCAACCTCAG GACTGAGCAT GCTGGGCATT 39750
GTGGGGTCCG AAGGTGGCTC CCCTGTCCCC TTCAAAATAC CCTCTTTTTC 39800
TTTTCTTCTT TTTTTTTTTT TTTTTTTTTT GAGACGAAGT CTTGCTCTGT 39850 | REPEAT
TGCCCCAGCT AGAGTGCAGT GGTGCGATCT CAGCTCCCCG CAACCTCTGC 39900
TTCCCGGGTT CAGGCGATTC TCCTGCCTCA GCCTCCTGAG TAGCTGGGAT 39950
TACAGGTGCC CACCGCCACG GCTGGCTAAT TTTTGTATTT TTAGTAGAGA 40000 | var(39970):[A:0.30]
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AACCTGCCCA CCTCAGCCTC CCAAAGTGCT GGGATTACAG GTTTGAGCCA 40100 | var(40062):[G:0.11]
CTGGGCCTAG GCCGTTTTTT TTTTTTTTTG AGAGGGAGTC TCACTCTGTT 40150 | REPEAT
GCCCAGGCTG GAGTGCAATG GCGCGATCTT GACTCACTGC AACTCCATTT 40200 | var(40174):[T:0.01]
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CAGGTGCATG CCACCACGGC CAGCTAATTT TGTATTTTTA GTAGAGACAG 40300
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CTGCCCGCCT TAGCCTCCCA AAGTGTTGGG ATTACAGGTG TGAGCCACCG 40400
CGCCCAGACC AAAATATGCT CATTTTAATA AAATGCACAA GTAGGTTGAC 40450 | var(40438):[C:0.40]
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GCCCTCCCCT AAAGACTCAT CAATGTGAGG CTCAAGAACC TTCTTAGGCT 40550 | var(40527):[A:0.05] | REPEAT
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CCGTGACCAC CGTCCTCTGC CTGCTCCATT TCTTGGTGGC CTTCCTTTAG 40850 | var(40812):[A:0.01]
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                                  A  L  G   D  V  A   776
GCAGAGGAAA TGAGAAGAAG CCCAGTAGCG TGAGGGCTCT GTCCATTGTC 40950
G  R  G  N   E  K  K   P  S  S   V  R  A  L   S  I  V  793
CTCCCCATCG GTAAGCGCGG GCCGGTCCCC CAGCGTCCCC CAGGTCACAG 41000
 L  P  I                                              796
CCTCCCGCTA TGTGACCTCG TGCCTGGCTG GTTGGGCCTG TTCACTTTTT 41050
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GCGCGCTCTC CCTGCCTCAC CCATGCTCAT CCTAACTCGG TTACCATCAC 41300
ATCTCTTTTT TCTTTTTTTC TTAAATTTTA AGAAAAAAGA AATTTAATTT 41350 | REPEAT
TTTTGAGAGA CAGAGTCTTG CTCTGTCACC CAGGCTGGAG TGCAGTGGCA 41400 | var(41381):[-:0.16]
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CCTCAGCCTC CTGAGTAGCT GGTGCAAGCC ACTATACCCC ACTTCCTATT 41500
TCTTAAAAAG TCACAGCCCT GTGTGTGGCT AATCCTGGAC AGAAATCTAG 41550
AAGAAGTCAG CTACTTCTGG GGCGTGGCTC ACCCAGTGGG CTTCAGGTTA 41600
GATATTTCTT ATACTTATGA GGCTGGGTGT GGTGGCTTAT GCCTGTAATC 41650 | REPEAT
CCAGCACTTT GGGAGGCTGA AGTGGGTGGA TTGCTTGGGC TCAGGAGTTC 41700
GAGACCAACC TGGGCAACAT GGCGAAACCC TGTTTCTAGA AAAGGTACAA 41750
AAATTAGCTG GGCAGGTGGC ACGTGCCTGT GGTACCAGCT ACTTGAGGGC 41800
CTGAGGCAGG AGGATCGCTT GAACCTGGGA GGTCGAGGTT GCAGTGAACT 41850 | var(41817):[A:0.14]
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AAAAAAAAAA AAAGAAAGAA AAAAATTCTT ATGCATAGAT TTGCCTCTTT 41950 | REPEAT
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TCTGTTTGTT TGTTTTGAGA TGGAGTCTCG CTCTGTCGCC CAGGCTGGAG 42000
TACAGTGGCT CAACCTCGGC TCACTGCAAC CTCTGCCTCC CGGGTTCAAG 42050
CAATTCTCCT GCCTCAGCCT CCTGAGTAGC TGGGACTACA GGCGCCCGCC 42100
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TCAGCCTCCC AAAATGCTGG GATTACAGGC GTGAGCCACC AGGCCCAGGC 42250
CGCAAGGCGA TCTCTAAACA AACATAAAAG ACCAGGAGTC AAGGTTATGG 42300 | var(42252):[A:0.21]
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GGGGCAGCTG TGTGACAGAG CGTGCCTCTC CCTACAGTGC TCCTCGTCTT 42400 | Exon 17
                                  V   L   L   V   F 801
CCTTTGCCTG GGGGTCTTCC TTCTATGGAA GAACTGGCGG CTTAAGAACA 42450
  L   C   L   G   V   F   L   L   W   K   N   W   R   L   K   N   817
TCAACAGCAT CAACTTTGAC AACCCCGTCT ATCAGAAGAC CACAGAGGAT 42500
I   N   S   I   N   F   D   N   P   V   Y   Q   K   T   T   E   D  834
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 E   V   H   I   C   H   N   Q   D   G   Y   S   Y   P   S         849
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GACAATGGCC TTGGTGGTGT AGAGGAATGT TGGATAAGCA AATAGAGAGC 42750
TCCATCAGAT GGTGACAGGG CAAAGAAAGT CAAAAGGAGT TCAGAGGCCG 42800 | REPEAT
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AACCCCGTCT CTATTAAAAA TACAAAAAAT TAGCCAGGCG TGGGAGTGGG 42950
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CCCGGGAGGC AAGAGCAAAA CTCCATCTCA AAAAAAAAAA AAAAAGGAGT 43100
TCAGAGGCCC GGCATGGTGG TTCACACATG TGATCCCAGA ACTTGGGGAG 43150 | REPEAT | NOT SCANNED
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GCACAGCAAG ACCCCATCTC TGCAAAAAAT AAAAATTTAG CCCAGTGTGG 43250
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TTGAGGCTAA GGTAGGAGAT TGAGACTGCA GTGACTTGTG ATTGCGTCAC 43350
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AAAAATTCAA AGAAGGGTTT CCAGAGGGCC AGGAGGGAGG AAGGGAGAGG 43450
AGGTGTTTTA TTTTTTTGCT TTTATTTTTT ATTTTGAGAC AGAGTCTCTC 43500 | REPEAT
TCTGTCACCC AGGTTGGAGT GCAGTGCTGT GATCTTGGCT CACTGCAACT 43550

TCTGCCTCCT GGGTTCAAGC AATTCTTATG CCTCAGCCTC AGCCTCCTGA 43600
GTAGCTGGGA TTACAACACT ATGCCCGGGT AATTTTTGTA TTTTTAGTAG 43650 | var(43627):[A:0.08]
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CCACTGCGCC CGCCTTGATC TTTACACAAG GGGTTTAGGG TAGGTAGCCT 43800 | REPEAT
TCTCTGAACC AGGAGAACAG CCTGTGCGAA GGCCCTGAGG CTGGACCGTG 43850
CCTGTTGGGT TTGAGGCCGT TGTAGCTGGA GCAAACAGAG AGAGGGGTAA 43900
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TACTCACCGT CTCCCTCTGG CGGCTCCTGG GGGAACATGC TTGGGGATCA 44050 | var(44009):[A:0.20]
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GGCAGAGACA GATGGTCAGT CTGGAGGATG ACGTGGCGTG AACATCTGCC 44200 | Exon 18 | UTR
      R   Q   M   V   S   L   E   D   D   V   A             860
TGGAGTCCCG TCCCTGCCCA GAACCCTTCC TGAGACCTCG CCGGCCTTGT 44250 | var(44240):[A:0.02] | var(44243):[A:0.21]
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CAGCCTGGTG CCCATCCTCC CGACCCCTAC CCACTTCCAT TCCCGTGGTC 45000 | var(44964):[G:0.21] | var(44994):[T:0.01]
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TTGACCGGGA CTTCAAAGCC GTGATCGTGA ATATCGAGAA CTGCCATTGT 45200 | var(45173):[C:0.08]
CGTCTTTATG TCCGCCCACC TAGTGCTTCC ACTTCTATGC AAATGCCTCC 45250
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AAGCCATTCA CTTCCCCAAT CTTGTCGTTG ATGGGTATGT GTTTAAAACA 45300
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GCGTGGTGGC GGGCACCTGT AGTCCCAGCT ACTCGGGAGG CTGAGGCAGG 45500
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CACTGCAGTC CGCAGTCTGG CCTGGGCGAC AGAGCGAGAC TCCGTCTCAA 45600 | var(45577):[T:0.07]
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GTTTGCACTT TGTATATTGG TTGAAACTGT TATCACTTAT ATATATATAT 46400
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GCCTGAATGT CTTACTGTGA TCAATTAAAT TTCTTAAATG AACCAATTTG 46700
TCTAAACTCG ATGCACGTTC TTCTGTTCGC GCGCTTCTTT TTGTTTTTTT 46750 | var(46728):[T:0.02] | var(46731):[T:0.01] | REPEAT
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GGCATGATCT CGGCTTACTG CAAGCTCCGC CTCCCAGGTT CAAGCAATTC 46850
TCCTGCCTCA GCCTCCCTAG TAGCTAGGAT TACAGGTGAG TGCCACCACG 46900
CCTGGCCAAT TTTTTTTTTT TTTTTTTTTT TGAGACAGAG TCTCGCTCTG 46950 | REPEAT | NOT SCANNED
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CCTCCCAGGT TAATGCCATT CTCCTGTCTC AGCCTCCTGA GTAGCTGGGG 47050
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GAGCCACCGC GGGTGGCCAA CGCTAATTTT TTTGTTTTTT TAGATGGAGT 47250 | REPEAT | REPEAT
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AAAAAAAGAA ATGAAGTCTT GATACGGTGG CTCATGCCTG TAATCCCAGC 48300 | REPEAT
ACTTTGGGAG GCCAAGGCAG GCGGATCACG AGCTCAGGAG ATCGAGACCA 48350
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GCCAGGCATG GTGGCGGGCG CCTGTAGTCC CAGCTACTTG GGAGGCTGAG 48450
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CGCGCCACTG CACTCCAGCC TGGGCGACAG AGCGAGACTC TGTCTCAAAA 48550
AAAAAACAAA AAAGAAATGA AAATCTCCCT GTCTGTCCTT AAGGACCTGA 48600
C
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