GENETIC VARIATION OF GENES RELATED TO HYPERURICEMIA AND GOUT IN THAI NCDS PATIENTS

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GENETIC VARIATION OF GENES RELATED TO HYPERURICEMIA AND GOUT IN THAI NCDS PATIENTS

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NISA MAKRUASI

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Genome-wide association studies (GWAS) have been conducted to investigate the differences between the genomic loci associated with urate concentrations and gout in the large populations in many countries. However, to date, there has been no information about the Thai population. This study aims to identify the new genetic predisposition of hyperuricemia and gout in Thai non-communicable disease (NCDs) patients. A pilot whole-genome sequencing (WGS) was performed on the genomic DNA of four male participants (Hyperuricemia, gout, early-onset gout, and normal) using the Illumina HiSeq X Ten platform (Macrogen, South Korea). The missense Ala17Thr (G>A) GLUT9 mutation was found only early- onset gout. The Pro1146Pro, A>G RREB1 mutation was found in hyperuricemia and gout. WGS also identified Ile223Ile (C>T) ABCC4 and Ala2872Thr (G>A) LRP2 mutation in hyperuricemia, early-onset gout, and gout, because a missense of LRP2 Ala2872Thr (G>A) is a novel finding mutation associated with hyperuricemia and gout. The frequencies of LRP2 Ala2872Thr (G>A) was investigated in patients with hyperuricemia, and NCDs by using polymerase chain reaction and a DNA sequencing analysis as a cross-sectional study. As results, the association between LRP2 rs2228171 in hyperuricemia. However, the results provided an association between LRP2 rs2228171 in hypertension.

Keyword : LRP2 rs2228171, Megalin, Serum urate level, non-communicable disease, polymorphism

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NISA MAKRUASI

TABLE OF CONTENTS

Page

TABLE OF CONTENTS

Page

FIGURES OF CONTENTS

Page

CHAPTER 1
INTRODUCTION

Background

Uric acid is a useless product from purine nucleotide metabolism in human. Uric acid can be eliminated by renal. Due to lacking of hepatic uricase enzyme (urate oxidase) in humans, uric acid cannot be changed to allantoin. So, serum uric acid can be elevated in patients [1, 2]. The prevalence of hyperuricemia is predominant in adult males (24-29 percent) more than adult females (2.6-20 percent). On the contrary, there is a rare prevalence in children [3-6]. During 2007-2008, the prevalence of hyperuricemia and gout in the USA increased from 3.9 percent to 21.9 percent, respectively [7]. Nowadays, the incidence of hyperuricemia is about 4.3-5 persons per 100,000 in the Thai population. Moreover, males have a high risk of progression to gout from hyperuricemia [8]. In addition, a report from Khonkaen province found 11.8 percent in prevalence of hyperuricemia [9].

The definition of Hyperuricemia described as serum uric acid over 7 mg/dL in females, and over 6 mg/dL in males [10]. Asymptomatic hyperuricemia can be evaluated by a high level of urate without signs and symptoms of inflammatory arthritis. About 10-30 percent of asymptomatic hyperuricemic patients can progress to gout [11]. Gouty arthritis, the most common inflammatory arthritis characterized by swelling, redness, and pain around a joint, is originated from the accumulation of urate crystals that is called monosodium urate (MSU) in the joint space [12]. There are several criterias for a diagnosis of gout are utilized [10, 13-15]. Currently, the criteria of the American College of Rheumatology/ European League Against Rheumatism collaborative initiative (ACE/EULAR) 2015 is generally used [16, 17].

Several studies identified the association between hyperuricemia and diabetes mellitus type 2 (DM2), metabolic syndrome [18-23], and cardiovascular disease [20-29]. Although evidence of renal destruction causing hyperuricemia has not been demonstrated [30], some cohort studies reported the relationship of serum uric acid and risk of chronic kidney disease progression [24, 26, 28, 31-34].

In contrast, some studies have shown that there was no association between hyperuricemia and other diseases as mentioned above[35, 36]. Several studies were found that 30-50 percent of asymptomatic hyperuricemic patients have urate deposits in joint or tendon detected by ultrasonography [37-40]. Moreover, there was a lot of monosodium urate crystal accumulated on the cartilage surface in people with prior history of gout [41].

Hyperuricemia is the imbalance of endogenous production and excretion of uric acid [42]. There are rare diseases caused by their inherited diseases involving enzymes in purine metabolism abnormality (X-linked superactivity of PRPS1 and defect of hypoxanthine-guanine phosphoribosyltransferase [HGPRT]) [42]. Currently, it has been claimed that several factors affect the level of serum uric acid, for example, genetics, obesity, alcohol, and smoking [23, 43-48]. Moreover, many studies have been reported the association between genetic and serum uric acid ranged from 25 to 63 percent [5, 6, 49]. The production of uric acid is an average of 600-800 mg per day in the body. In the normal physiologic pH (pH=7.4), about 98 percent of uric acids are in the form of "urate anion" [50, 51]. Urate transporters that locate on renal proximal tubules are important to excrete the urate [51]. The deficiency of enzymes in purine metabolism leads to hyperuricemia [52]. Furthermore, limitation of excretion of urate and/or decline renal function can cause precipitate of urate crystal in joint spaces and other tissues leading to a gouty attack. Over 90 percent of gout patients are caused by under excretion [12, 53] Contrastly, the cause of hyperuricemia from overproduction is only found in 10 percent [52]. So, urate homeostasis involved the balance of production, excretion, and reabsorption of urate [54].

Genome-wide association studies (GWAS) have been developed and used to be an important tool for a detect the association between genome and hyperuricemia including gout [55]. GWAS studies reported the importance of urate transporters. So, the studies of single nucleotide polymorphisms (SNPs) of many groups of genes including SLC22A12, SLC2A9, and ABCG2 are encoded to urate transporters URAT1, GLUT9, and ABCG2, respectively. Thus, urate transporters are necessary to transport urate and to maintain homeostasis [55]. All of the urate transporter genes have been reported in patients

with hyperuricemia and gout in foreign countries [56]. Dysfunction of some urate transporter genes can increase the level of serum uric bring about gout. Moreover, the largest GWAS that studied more than 140,000 subjects of a European country has been found 28 genetic loci associated with serum uric acid and gout [56].

Greater understandings of SNPs of genes related to urate have led as important tools to identify the risk that develop hyperuricemia. Moreover, it can find a biological marker, construct an innovation to detect and predict the risk of gout in a patient with asymptomatic hyperuricemia, and use appropriate medication for gout patients.

Accordingly, the study is aimed to determine, and identify genetic associated with hyperuricemia and gout in Thai NCD patients.

CHAPTER 2
REVIEW OF THE LITERATURE

The review of the literature was included biosynthesis and degradation pathway of uric acid, types of hyperuricemia, urate transporters, genetic associations with urate levels, SNPs, and methods to detect SNPs.

2.1 Biosynthesis and degradation pathway of uric acid

Purine metabolism: purine is a building block of DNA and RNA, components of nucleosides such as adenosine and guanine. Adenosine and guanine are important factors for ATP, GTP, cyclic AMP, S- adenosyl methionine, nicotinamide adenine dinucleotide (NADH), and nicotinamide adenine dinucleotide phosphate (NADPH). Purine and its derivatives comprise adenine, guanine, isoguanine, hypoxanthine, xanthine, theobromine, caffeine, and uric acid (as shown in Figure 1) [57]

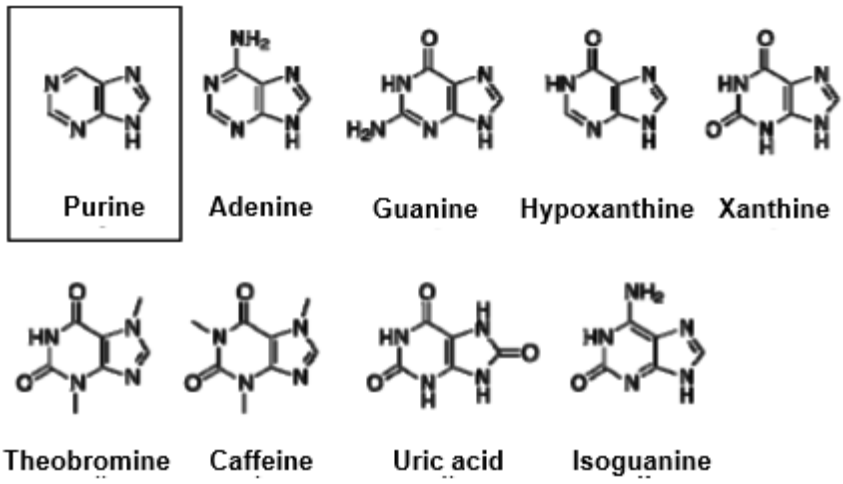

Figure 1 Chemical structures of purine and its derivatives [57]

Uric acid is a waste product from the metabolism of purine nucleotides and excretes via the kidney. Purines are produced from dietary riches purines, de novo biosynthesis pathways, and salvage pathways of purine nucleotides. There are biosyntheses, and degradation pathways of metabolism of purine nucleotides, as described following.

Biosyntheses pathways are occurred in liver cells, comprising of de novo synthesis and salvage pathway, respectively [58, 59]. For de novo synthesis, the synthesis of nucleotides from phosphoribosyl pyrophosphate(PRPP) is a precursor of nucleotides. The first step is to use PRPP synthetase (ribose-phosphate pyrophosphokinase 1) and PRPP Glutamyl amidotransferase, which is a rate-limiting enzyme to control the rate of synthesis. There are 11 steps in the process until the first purine nucleotides, inosine monophosphate (IMP). The IMP is processed until receive adenosine monophosphate (AMP), and Guanine monophosphate (GMP), respectively. For the salvage pathway, this is a recycling of degradation product of purine nucleotide backs to build new purine nucleotides. The salvage pathway is used less energy, and a short step of the pathway. Hypoxanthine- guanine phosphoribosyl transferase (HGPRTase), and adenine phosphoribosyltransferase (APRT) are necessary enzymes in this pathway. HGPRTase is used for the synthesis of IMP, and GMP. APRT is used for the synthesis of AMP. Feedback inhibition is a regulation of biosynthesis [58], rate-limiting enzymes are controlled by-products of reactions. For example, PRPP synthetase is inhibited by ADP and GDP. Next, amidophosphoribosyltransferase that can excite by PRPP, is inhibited by its product. Another way, feedback inhibition occurs in the junction of the reaction of IMP to AMP or GMP.

Uric acid is an end product from the degradation of purine nucleotides[58, 59]. Uric acid metabolized in the liver and excreted via the kidney (Figure 1, 2). In most mammals, purine is transformed into uric acid, and subsequent to allantoin by uricase enzyme, respectively. Allantoin can pass through the bloodstream and filtrate in the kidney. Due to lacking of uricase enzyme in humans, so uric acid cannot be converted to allantoin, (Figure 2). Figure 2 is shown purine catabolism, uric acid formation. Pathways of metabolism include reactions catalyzed by different enzymes such as AMP deaminase, adenosine deaminase, 5’-nucleotidase, purine nucleoside phosphorylase, guanine deaminase, and xanthine oxidase. So, a higher level of uric acid is also found in the serum of human [57].

The level of uric acid in serum depends on the rate of synthesis in the liver, degradation of purine nucleotides, and renal excretion of uric acid [60]. Renal excretion is an important factor for uric acid. More than 90 percent of patients with hyperuricemia defects in renal excretion [61].

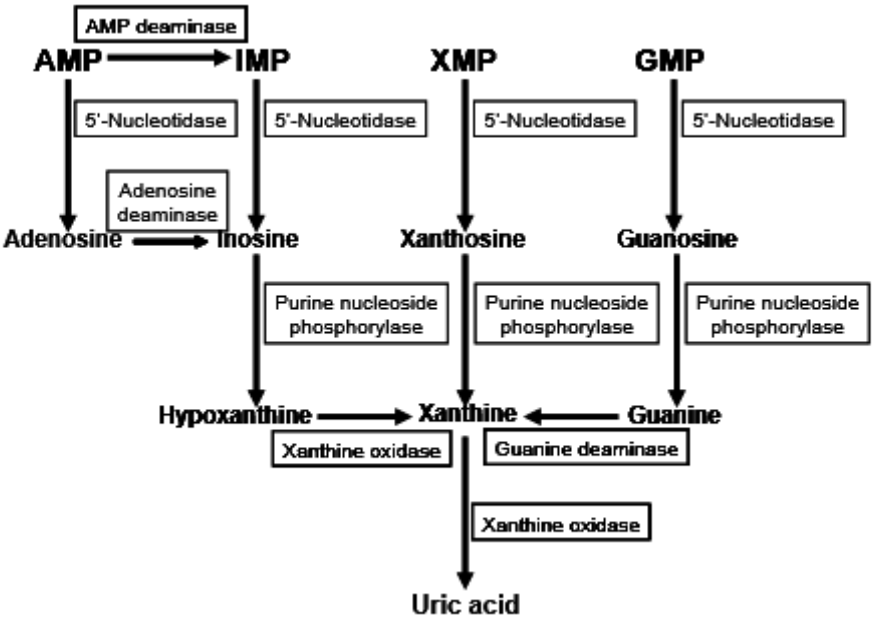

Figure 2 Metabolic pathways of uric acid formation [57]

Seventy-five percent and 25 percent of uric acid are excreted via renal, and extra-renal (gastrointestinal tract), respectively. Free form of uric acid is filtrated via glomerulus, most of them are reabsorbed in proximal tubule via urate transporter. Ten percent of uric acid is secreted in the late proximal tubule to filtrate again [62]. Dysfunction of urate transporters is a common cause of renal excretion of uric acid [63-65].

2.2 Types of hyperuricemia

Types of hyperuricemia are classified by urate excretion and urate clearance of renal [66]. There are categorized into overproduction, under-excretion, and combine types. (Figure 3). The overproduction hyperuricemia group is defined by urate urine excretion (uuE) more than 25.0 mg h^{-1} / 1.73 m^2 (600 mg per day/ 1.73 m^2). Underexcretion

hyperuricemia group is defined by fractional excretion of urate (FEuA) less than 5.5 %. The combined type is defined by meeting the criteria of overproduction and underexcretion hyperuricemia. The normal type is defined by uuE less than 25 mg h^{-1}/1.73 m^2 and FEuA is more than 5.5%. uuE unit, mg h^{-1} /1.73 m^2, respectively [67].

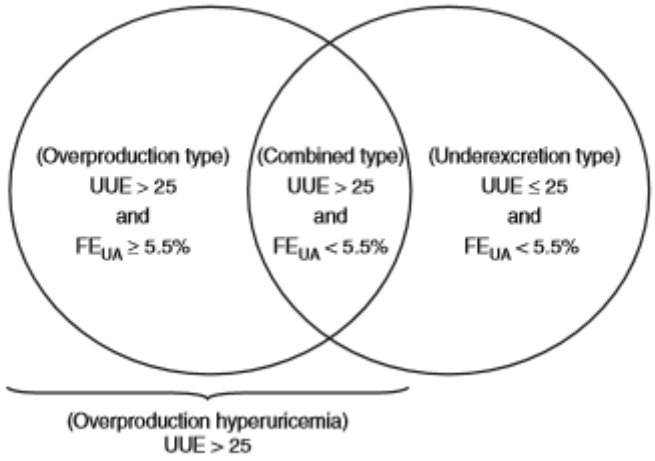

Figure 3 Classification of hyperuricemia depends on uuE and FEuA [67]

Recently, Ichida K et al (67) showed a new model to classify hyperuricemia. Extra-renal urate excretion is added to the model. The model is categorized into renal overload and under-excretion types. Renal overload type is classified as overproduction and extra- renal under- excretion types (Figure 4). The current classification of hyperuricemia is categorized to urate 'overproduction type' (A), 'renal under-excretion type' (B), and the combined type. The new classification is categorized as 'renal overload type' (A), and 'renal under-excretion type' (B). Subtypes of renal overload are categorized as 'overproduction' (A1) and 'extra-renal under-excretion' (A2) [67].

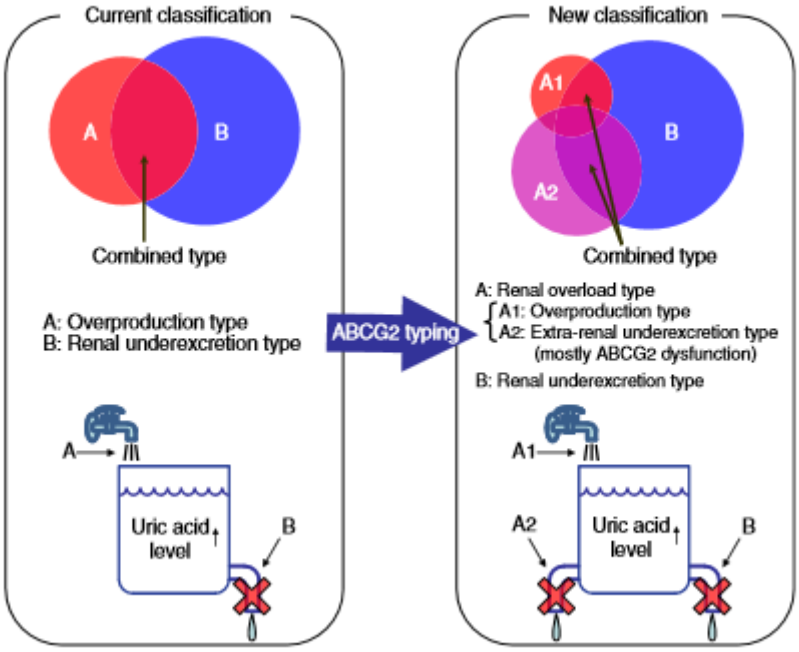

Figure 4 Model of pathophysiology and classification of hyperuricemia [67]

2.3 Urate transporter

Uric acids in blood vessels are freely filtrated via the glomerulus. Most of the uric acids are reabsorbed by using urate transporters protein that locates on the proximal tubule. Ten percent of uric acid is secreted and filtrated at the late proximal tubule [62, 64]. Therefore, 75% of uric acid is excreted via the kidney. More than 90% of patients with hyperuricemia are caused by dysfunction of urate transporter protein excreting uric acid [61]. Later, Genome-wide linkage scans [68-71] and Genome-wide association studies (GWAS) [46, 72-76] are studied, the objective of the studies is analysis the role of urate transporters such as a solute carrier protein 2 family and member 9 (SLC2A9 that control uric acid in serum.

The function of urate transporters is to control uric acid via the renal proximal tubule. (Figure 5). Uric acid is freely filtrated via glomerulus. Then, it is reabsorbed via a urate transporter at the renal proximal tubule. After that uric acid is excreted in urine by different urate transporters. In figure 5, the solid line is demonstrated in the reabsorption of uric acid. Dotted is demonstrated in the secretion of uric acid [57].

Urate transporters are regulated by genes that encode urate transporters. Dysfunction of genes involving urate transporters can lead to hypouricemia, hyperuricemia, and gout. Genes that associate with urate transporter are classified as urate reabsorptive, and urate excretion transporters, respectively. SLC22A12 (URAT1) and SLC2A9 (URATv1/GLUT9) are identified as the main control of the reabsorption of uric acid (73-75, 77-79). But SLC22A6 (OAT1), SLC22A8 (OAT3), SLC17A3 (NPT4), ABCC4 (MRP4), and ABCG2 (BCRP) are involved renal secretion [63, 80-83] (Figure 5).

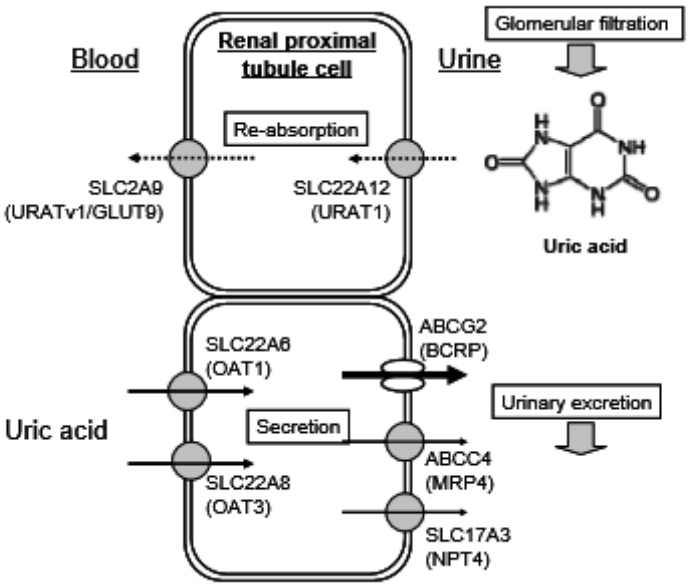

Figure 5 Renal reabsorption and excretion of uric acid [57]

2. 3. 1 Urate reabsorptive transporters include urate anion transporter 1 (URAT1), Organic anion transporter 4, and Glucose transporter 9, respectively.

2.3.1.1 Urate anion transporter 1 (URAT1) is encoded from SLC22A12 (solute carrier family 22 (organic anion/ cation transporter), member 12). It is located on the long arm of chromosome 11 (11q13.1) that is discovered in 2002 [77]. URAT1 is located on the luminal side of the proximal tubular epithelial cell membrane. URAT1 can interchange uric acid with Cl- or organic anions in epithelial cells through proximal tubular lumen[77]. URAT1 is a protein that comprises 12-transmembrane domains and 555 groups of amino

acid residue [84, 85]. URAT1 is a key to control the hemostasis of uric acid in the blood, it can reabsorb urate from the proximal tubule back to epithelial cells. Many studies were found that drugs such as probenecid, sulfinpyrazone, and benzbromarone interfere with the function of URAT1, and subsequently increase uric acid excretion [86]. Several drugs have an influence on the level of uric acids such as valproic acid, fenofibratet, losartan, and trimethoprim [87-89]. Angiotensin II receptor blockers such as losartan can affect URAT1 by increase uric acid excretion, and decrease the blood level of uric acid, respectively [90]. Losartan can suppress URAT1 and lead to decrease the level of uric acid in hypertensive patients [87, 91].

The expression study of the URAT 1 gene in the animal model was found predominant in males than female mice, ratio 2.3:1 [85]. The previous report was shown that testosterone could increase the promoter activity of the human URAT1 gene [92].

Many studies reported 25 mutations of the URAT1 gene that affect disease [62, 77, 93-100]. Other studies reported 5 variants of URAT1 that were gout phenotype, one variant increased FE-UA [101], and 2 variants were associated with decreasing FE_{UA} [102, 103]. Mutation of SLC22A12 that translates to URAT1 transporter was reported in the majority of idiopathic renal hypouricemia [77, 93, 94].

2.3.1.2 Organic anion transporter 4 (OAT 4) is encoded from SLC22A11. OAT 4 is located on the apical membrane of epithelial cells [104, 105]. OAT 4 is important in the process of reabsorption of urate by using an intracellular dicarboxylate gradient [106].

2.3.1.3 Glucose transporter 9 (GLUT 9) is encoded from SLC2A9 that located on the short arm of chromosome 4 (4p15.3-p16) [107]. GLUT9 is a member of glucose transporter (GLUT) [108]. The structure of GLUT9 is a type II GLUT isoform that comprises 12 domains of transmembrane. There is a huge extracellular loop, amino and carboxyterminal end on the cytoplasmic side between transmembrane domains 1 and 2 [109].

Firstly, GLUT 9 was defined as glucose and/or fructose transporter but now it is known as urate transporter [106]. A genome-wide perspective study was demonstrated the important role of SLC2A9 to control the level of uric acid, it could explain

about a 3.5 percent change in the level of uric acid [72]. Later, a study was found a relationship between single nucleotid polymorphism (SNP) in SLC2A9 gene and the level of uric acid in the serum [110]. Another study was shown that mutation of SLC2A9 leading to dysfunction of GLUT9 was correlated with hypouricemia caused by the reduction of reabsorption of uric acid [111]. Knockout systemic GLUT9 in mice study was reported to inhibition of reabsorption of uric acid [112, 113]. Several studies detected the relation between GLUT9 and serum uric acid [73, 74, 78]. Moreover, transporter proteins such as ABCG2, NTP1, and NPT4 are located on late proximal tubule, the function of the transporter proteins are to secrete uric acid [114].

Genome-wide association studies (GWASs) from foreign countries have reported the relationship between SLC2A9 and level of uric acid [72, 74-76, 78, 115-118], excretion fraction of uric acid, gout, and body mass index (74, 75, 119). However, the study in the African, American, and European population have not shown a correlation between single nucleotide polymorphisms (SNPs) and gout [101].

Sperling O.(1992) [120] defined hereditary renal hypouricemia. It is an inherited defect of urate transporter protein that locates on proximal tubular cells. Renal hypouricemia is defined as the level of serum uric acid less than 2.0 mg/dl and no renal or systemic diseases such as Fanconi syndrome, Wilson disease, and drug-induced tubulopathy. Renal hypouricemia is classified as dysfunction of the SLC22A12 gene (URAT1) as Type 1[77, 93-96], and SLCA9 (GLUT9) as Type 2 [79, 121]. Tasic V, et al. (2011)[62] reported the clinical characteristic and function of URAT1 variants, dysfunction of URAT1 can cause hypouricemia, and renal stone due to increasing the excretion of uric into the urine, and develop a serious complication such as acute renal failure. Moreover, the study was found an association between heterozygous missense mutation of SLC22A12 in 32 Macedonian and British populations that presented with hypouricemia.

2.3.2 Tubular secretion of uric acid transporters is comprised of a urate transporter, multidrug resistance protein 4 (MRP4), and ABCG-2.

2.3.2.1 Urate transporter (UAT). It is located on the brush border of the renal tubular epithelial cells of the rat. Leal-Pinto E. et al. (1997)[122] found that UAT has 322

amino acids and the function of UAT involved urate efflux from systemic cells and electrogenic urate transporter. Uricase inhibitors such as adenosine , oxonate, and pyrizinamide have been found to inhibit the activity of UAT channel [106].

2.3.2.2 Multidrug resistance protein 4 (MRP4/ABCC4). It is in the role of urate secretion in the proximal tubule [123]. MRP4 is located on HEK293 cells in human [124], and promote ATP-dependent urate extrusion from the cell into tubule lumen that resulting urate excretion [81, 125]. Contrastly, Hoque MT et al. (2009)[126] reported that organic anions like cAMP, cGMP, and methotrexate can be secreted in tubule lumen via MRP4. Human MRP4 is inhibited by benzbromarone and probenecid, uricosuric drug [124].

2.3.2.3 ABCG2 It is an ATP-binding cassette of half-transporter protein. ABCG2 is located on chromosome 4, that is (4q22)(127) or 4q25 [128].

Urate transporter protein, ABCG2 is a high-capacity transporter to excretion of uric acid via the kidney, liver, and small intestines [63, 129]. The first study that analyzes the association of uric acid and SNPs was reported by Dehghan et al (2008)[72]. Genome-wide association studies (GWAS) were reported the mutation of ABCG2 causing hyperuricemia [130, 131]. Nonsynonymous SNP in the ABCG2 Q141k study was found a negative effect on the function of ABCG2 [124].

Analyses of the human genome and in an animal model found that abnormal function of ABCG2 could be caused by gout. Several genome-wide association studies (GWASs) identified the relation of uric acid and urate transporters including ATP-binding cassette, subfamily G, member 2 (ABCG2) [56, 72, 115, 132-134]. Furthermore, functional studies found significant biological mechanisms and pathophysiology of ABCG2 [63, 67, 131]. The analyzing of ABCG2 variants in Western and Asian populations were reported [135, 136]. Previously, GWASs of SNPs of ABCG2 and SLC2A9 with gout were reported in the European population [116, 117]. However, the study was done on African, American, and European individuals who have been found no association between SNPs and gout [101].

2.4 Genetic associations with urate levels

As mention above, the genetic variations of urate transporter are leading to hyperuricemia and gout. The large GWAS was studied by the Global urate Genetic Consortium (GUGC), the subjects of European ancestry had more than 140,000 subjects, and the study found that 28 genetic loci with association to serum uric acid and gout [56]. The genetic location related to serum uric acid level and gout is shown in Tables 1 and 2.

Table 1 Genetic associations with serum urate and gout [137]

Location	Gene	Protein	Genetic association with gout/hyperuricemia
4q22.1	*ABCG2*	BCRP	Multiple common variant associations with serum urate levels, renal under-excretion gout, and overall risk of gout[56, 115-118, 132, 138-143]
4p16.1	*SLC2A9*	GLUT9	Multiple common variant associations with serum uric acid levels, renal overload gout, renal under-excretion gout, and overall risk of gout. Low-frequency variants associated with renal hypouricemia type 2 [56, 115-118, 138, 139, 141-143]
6p22.2	*SLC17A1*	NPT1	Multiple common variant associations with serum uric acid levels and gout risk [56, 118, 138, 140, 143-146]
6p22.2	*SLC17A3*	NPT4	Multiple common variant associations with serum uric acid levels. Rare loss-of-function variants found in patients with hyperuricemia [56, 140, 147]
11q13.1	*SLC22A12*	URAT1	Multiple common variant associations with serum uric acid levels, renal overload gout, renal under-excretion gout, and overall risk of gout. Low frequency variants associated with renal hypouricemia type1 [56, 77, 95, 98, 99, 115, 138, 141, 143, 148]

Table 2 Genetic association with serum urate [137]

Location	Gene	Protein	Genetic association with serum urate levels
2q22.3	*ACVR2A*	Activin A receptor type 2	Common variant association with serum urate levels[56]
10q11.2	*ASAH2*	N-acylsphingosine amidohydrolase 2	Common variant association with serum urate levels[56]
17q23.2	*C17ORF82*	Chromosome 17 open reading frame 82	Common variant association with serum urate levels [56]
2p23.3	*GCKR*	Glucokinase regulator	Common variant association with serum urate levels [56]
17q22	*HLF*	HLF, PAR bZIP transcription factor	Common variant association with serum urate levels [56]
8q21.13	*HNF4G*	Hepatocyte nuclear factor 4 gamma	Common variant association with serum urate levels [56]
15q26.3	*IGF1R*	Insulin-like growth factor 1 receptor	Common variant association with serum urate levels [56]
2q14.2	*INHBB*	Inhibin beta B subunit	Common variant association with serum urate levels [56]
12q13.3	*INHBE*	Inhibin beta E subunit	Common variant association with serum urate levels [56]
2q31.1	*LRP2*	LDL receptor related protein2	Common variant association with serum urate levels [115]

Table 2 (continued)

Location	Gene	Protein	Genetic association with serum urate levels
11q13.1	LTBP3	Latent transforming growth factor beta binding protein 3	Common variant association with serum urate levels [56]
16q23.2	MAF	MAF bZIP transcription factor	Common variant association with serum urate levels [56]
7q11.23	MLXIPL	MLX interacting protein like	Common variant association with serum urate levels [56]
3p21.1	MUSTN1	Musculoskeletal, embryonic nuclear protein 1	Common variant association with serum urate levels [56]
16q22.1	NFAT5	Nuclear factor of activated T-cells 5	Common variant association with serum urate levels [56]
15q24.2	NRG4	Neuregulin 4	Common variant association with serum urate levels [56]
1q21.1	PDZK1	PDZ domain containing 1	Common variant association with serum urate levels [56, 149]
1q22	PKLR	Pyruvate kinase, liver, and RBC	Common variant association with serum urate levels [56]
7q36.1	PRKAG2	Protein kinase AMP-activated non-catalytic subunit gamma 2	Common variant association with serum urate levels [56]

Table 2 (continued)

Location	Gene	Protein	Genetic association with serum urate levels
17q25.1	*PRPSAP1*	Phosphoribosyl pyrophosphate synthetase associated protein 1	Common variant association with serum urate levels [56]
12q24.13	*PTPN11*	Protein tyrosine phosphatase, non-receptor type 11	Common variant association with serum urate levels [56]
6p24.3	*RREB1*	Ras responsive element binding protein 1	Common variant association with serum urate levels [56]
10q21.2	*SLC16A9*	Solute carrier family 16 member 9	Common variant association with serum urate levels [56]
11q13.1	*SLC22A11*	OAT4	Multiple common variant associations with serum uric acid levels [56, 138, 139, 141]
8p21.2	*STC1*	Stanniocalcin 1	Common variant association with serum urate levels [56]
5q13.2	*TMEM171*	Transmembrane protein 171	Common variant association with serum urate levels [56]
6p21.1	*VEGFA*	Vascular endothelial growth factor A	Common variant association with serum urate levels [56]

Although hyperuricemia can be causing gout, several factors such as age, diet, drugs, and genetics are related to the level of serum uric acid [150, 151].

Hypouricemia defines as serum uric acid less than or equal to 2 mg/dL. About 0.8 percentage of inpatients and 0.2% of general people were reported in hypouricemia [5]. However, the prevalence of hypouricemia is high than current data but no diagnosis [152]. Hypouricemia can occur from reduction of fractional excretion of urate and elevation of xanthine excretion [153]. The lower level of uric acid is related to high fractional excretion of uric acid, caused by the mutation of genetics such as SLC22A12 (URAT1) [77, 154], and SLC2A9 (GLUT9) [75, 99] or drugs such as uricosuric or renal tubulopathy and neoplasms [5, 154].

Previously report the renal stone and exercised-induced renal failure in patients who have mutations of SLC22A12 (URAT1), and SLC2A9 (GLUT9), but have been not found in XDH (XO) genes [155, 156].

2.5 Single nucleotide polymorphisms (SNPs) and screening methods.

A single variant of DNA sequence can lead to SNP. The variation of genes presents when a single nucleotide (A, T, C, or G) vary from the members of species [157]. It must occur in at least 1 percent of the population [158] (Figure 6). Contrast to mutation, it is found in less than 1 percent of the population. Most SNPs are found outside of coding sequences. SNPs found within a coding sequence alter the function of a protein.

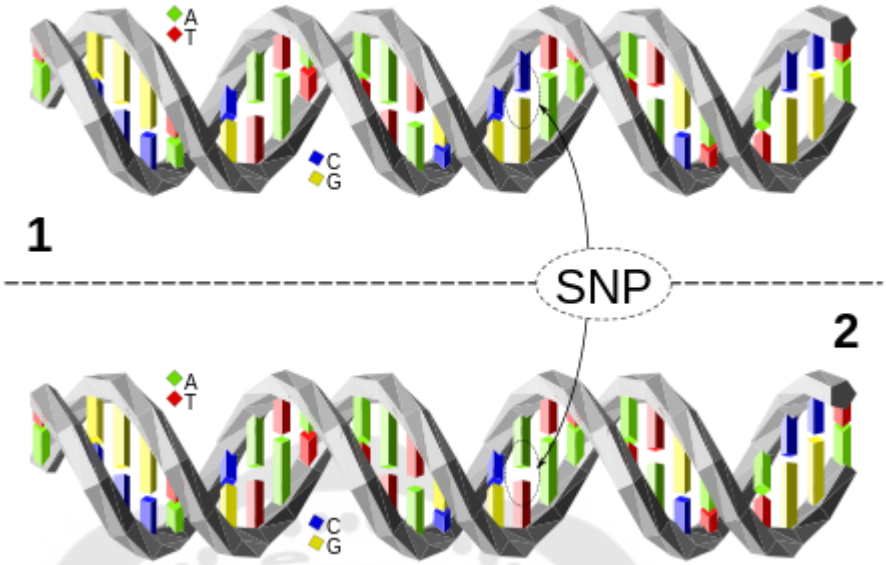

Figure 6 Location of SNP [157]

The location of SNPs is classified into inside and outside coding sequences. Outside coding sequence such as a promoter, 5' untranslated region (5' UTR), 3' untranslated region (3' UTR), exon-intron splicing, conserved noncoding sequence. Types of SNPs are categorized into non-coding regions and coding regions [157] (Figure 7). The coding region is classified as synonymous and non-synonymous. Synonymous means the only change of nucleotide but no effect on the protein sequence. But, non-synonymous means change of nucleotide that leads to the change of amino acid sequence, that occur missense and nonsense. Missense means SNPs on codon that translate to amino acid code resulting in protein non-function. Nonsense means point mutation in DNA sequence leading to stop codon and nonfunctional protein.

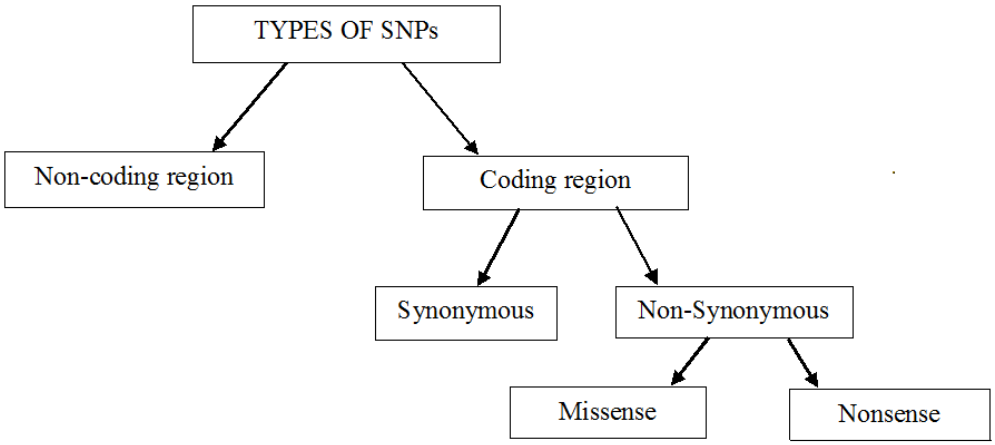

Figure 7 Type of SNPs [157]

SNPs may cause diseases such as cystic fibrosis, β-thalassemia, and sickle cell anemia [159-161]. Moreover, SNPs can be a biological marker to detect the risk of disease such as the apolipoprotein E gene associated with Alzheimer's disease [162], infectious diseases (AIDs, leprosy) neuropsychiatric disorder, dyslipidemia, diabetic type, stroke, cancer, gastrointestinal disease, and cardiovascular disease. Important, the variation of SNPs effect to pharmacogenetics [163] and the association of different drug metabolism [164-166].

The interest in SNPs is increasing leading to develop the method to detect SNPs such as single-strand conformation polymorphism (SSCP), restriction fragment length polymorphism (RFLP), PCR-based methods tetra-primer amplification refractory mutation system PCR (ARMS-PCR), real-time PCR with high resolution melting analysis (RT-PCR & HRM), and DNA sequencing.

The SSCP and RT-PCR & HRM have used the property of temperature melting and single-stranded conformation. These methods can identify specific alleles of SNP under the appropriate conditions for better results. The SSCP is widely used due to its simple technique, and inexpensive. The disadvantage of the method is low sensitivity because it depends on temperature. The sensitivity is decreased when sequences longer than 400 base pairs [167].

The RT-PCR & HRM is used as a melting curve analysis to detect the pattern of double-stranded DNA during heating. When the temperature increases, the double-strand break to single strand. We can detect a DNA-intercalating fluorophore (SYBR green), and demonstrate in the different graphs. The advantages of the method are high sensitivity, less expensive, easy to design probe. However, large samples must be processed in genotyping applications [168-170].

The RFLP method is uncomplicated and not difficult to do. It uses the restriction endonuclease enzyme to cut the specific restriction sites. The results are analyzed through a gel assay. The disadvantages of the method are required of specific endonucleases, use more than single experiments, and low throughput analysis[171, 172].

DNA Sequencing is the current method to detect SNPs. The first DNA sequencing using dideoxynucleotide triphosphates (ddNTPs) to terminate DNA synthesis is discovered by Frederick Sanger in 1977 [173]. The synthesis of DNA has used a 4 tubes reaction. Each tube is added primer to bind with DNA template, DNA polymerase, and deoxynucleotide triphosphates (dNTP) to the synthesis DNA strand, respectively. The different deoxynucleotide triphosphates (dNTP) such as ddATP, ddCTP, ddGTP, and ddTTP is added into reaction. The synthesis of DNA is stopped when ddNTP bind with complementary DNA. The reaction is analyzed by using polyacrylamide gel. The band of DNA is shown by using autoradiography and fluorescence dye [174]. The method can detect the sequencing between 500 and 800 base pairs. Later, shotgun sequencing is developed by cutting and overlapping pieces of DNA and then mapping DNA sequencing. It is better than the Sanger technique in the analysis of the large sequencing [175].

Currently, next-generation sequencing (NGS) including second and third-generation sequencing are developed. The second-generation sequencing (SGSTs)can analyze massively parallel sequencing for DNA library by using the template from PCR. Later the third-generation sequencing (TGSTs) can detect sequencing without using the DNA library. This technique can analyze each sequencing from single natural molecule sequencing, not synthesis in the lab. Thus the sequencing data is accurate, and not be

in error from synthesis by PCR. The advantage of TGSTs is large amounts of information, rapid, multiple coverages, no using de novo assembly, low price, a small quantity of DNA [175]. The favored SGSTs are MiSeq, 454/Roche, HiSeq/Illumina, Solexa, SOLiD/Life Technology, and Ion Torrent/Life Technology. The selection of SGSTs should depend on the type of research. The appropriate SGST to detect SNPs is HiSeq/ Illumina, respectively[176].

The Hiseq/Illumina has used the principle of synthesis [177]. The reversible terminated chemistry concept is applied to the machine [178]. Figure 8 is shown the steps of HiSeq/illumine sequencing. The principle of the method includes build a DNA library and use reversible dye terminators to identify sequencing. Make the DNA library (Figure 8-11), the step is following, first to prepare genomic DNA sample, make the DNA to small fragmentation around 200 to 600 base pairs, repair both ends of DNA to blunting end by exonuclease, and ligate the adapters to both ends of the fragments. Second, the single-stranded DNA fragments are bound into the surface (or flow cells), the surface is covered by oligonucleotide that compliments with adapter, leadint to the template DNA fixed on the flow cells. Third, bridge amplification by the PCR technique is done before denature the double-stranded molecules, and bring them to the next step [179]. The next step is to find sequencing by Illumina, by using the reversible terminator nucleotide technique as shown in Figure 10. To start the first cycle of sequencing, primer; all four labeled reversible terminator nucleotides; DNA polymerase enzymes are added to the flow cell. The nucleotides have chemical structures that protect from extension in the 3'OH end. So, the synthesis of DNA is stopped. Second, the uncoupled nucleotides are washed, and captured the image from fluorescence of each cluster. It records the first base for each cluster. Third, the chemicals that protect extension at 3' OH end are deleted. The reversible terminator nucleotide is used in the next cycle (Figure 10).

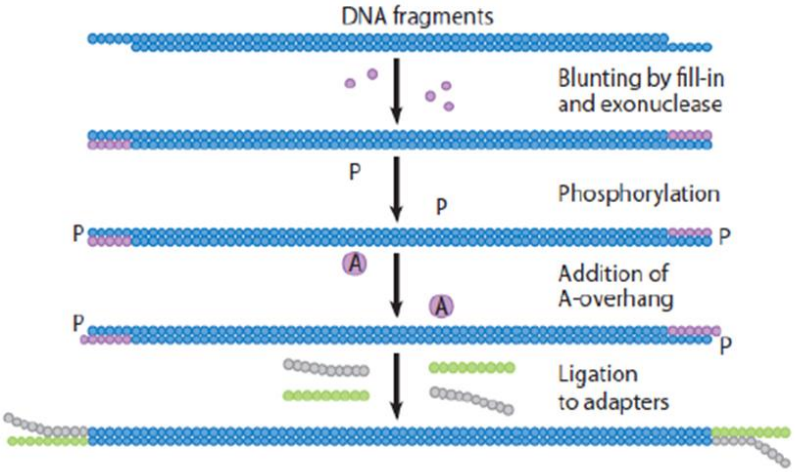

Figure 8 Prepare by DNA library by Illumina[180]

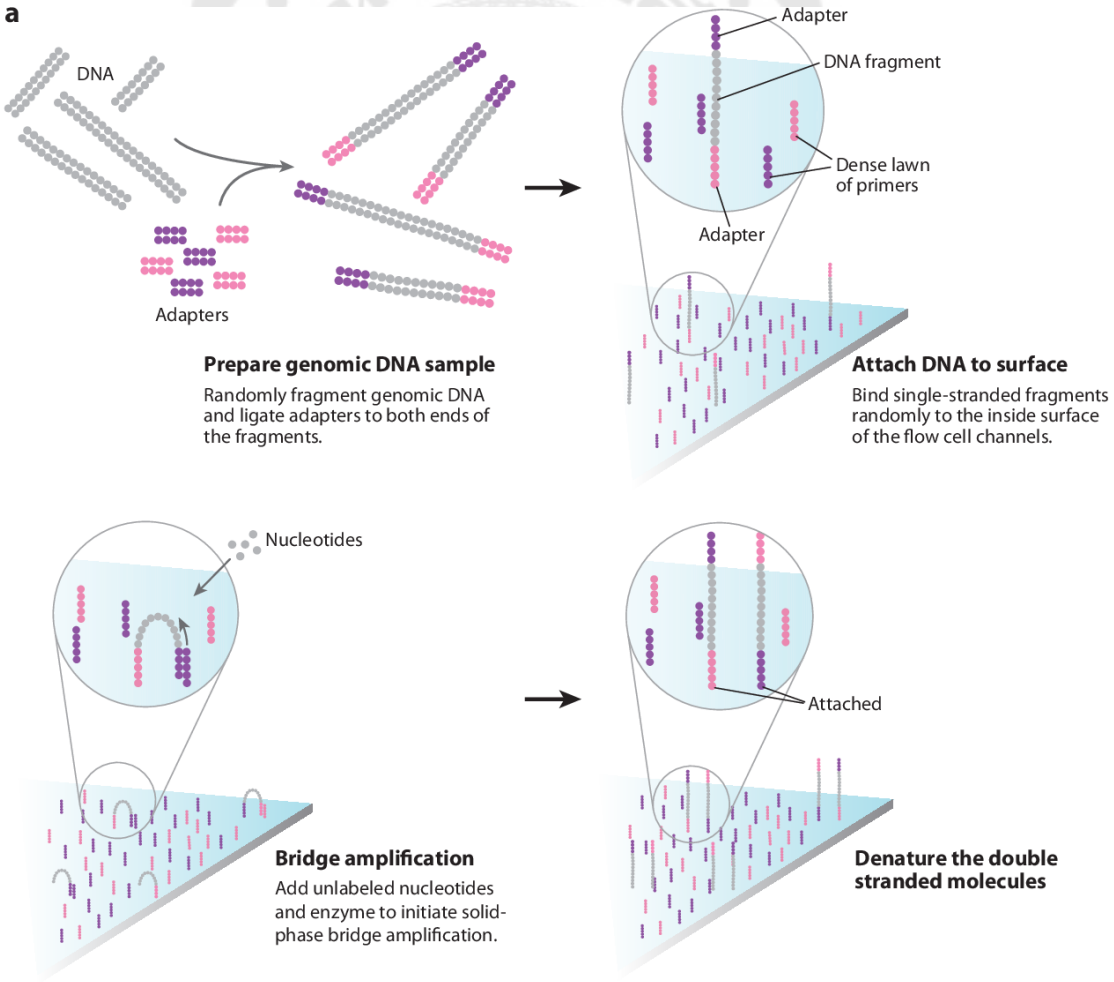

Figure 9 DNA library by bridge amplification [181, 182]

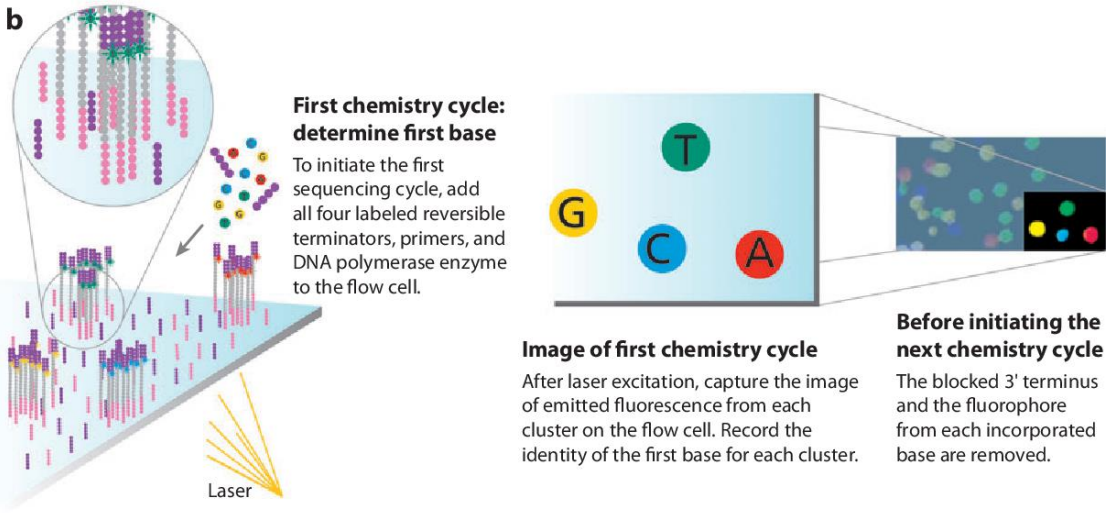

Figure 10 DNA synthesis and reversible dye terminators[181]

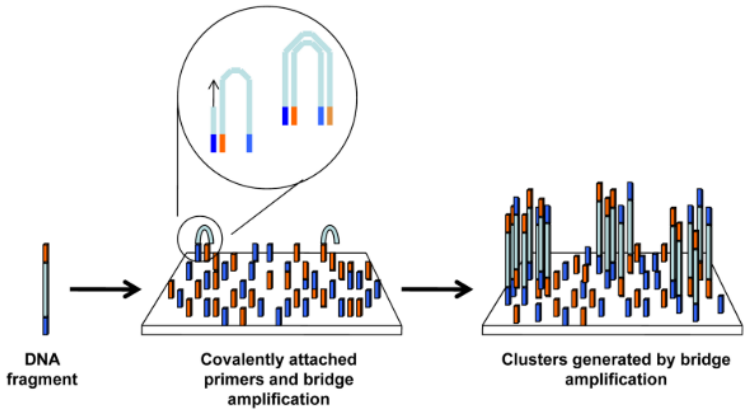

Figure 11 Solid-phase amplification used by Illumina sequencers[176]

Due to the DNA sequence by synthesis is analyzed base-by-base, it provides an accuracy of sequencing. It is appropriate to identify SNPs. Due to lots of DNA sequence, and short read is leading to high coverage. The DNA sequence can be aligned to the references and identified matches or difference in the sequence. Data from sequencing is shown in FASTQ or BAM [183].

CHAPTER 3
METHODOLOGY

In our study, the experiment was divided to phase I and II. The details of the phases were described below.

3.1 To Identify the new SNPS associated with hyperuricemia as phase I study

The objective of this phase was to identify new SNPs associated with hyperuricemia and gout.

3.1.1 Study design, subjects, and data collection

The study "Genetic variation of urate transporter genes in hyperuricemia and gout among Thai population (GUHGTHS)" by Tanunyutthawongse C, et al was conducted during 2017-2018 as cross-sectional study. Subjects with an age older than 25 years who health check-up in Srinakharinwirot university were included in the study. All participants with acute heart disease, kidney disease, and using anti-depressive drugs were excluded from the study. The study was approved by the review board and ethical committee of Srinakharinwirot University, Bangkok, Thailand (MEDSWUEC- 148/60E). Clinical profiles of our subjects including age, gender, comorbidities, blood pressure (BP), and body mass index (BMI) were collected. Blood chemistry including fasting blood sugar (FBS), serum uric acid (SUA), total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low- density lipoprotein cholesterol (LDL), triglyceride (TG) were collected from all participants.

In phase 1 of the study, we would like to find the new variants of genes affected with hyperuricemia. Thus we choose the 4 subjects from GUHGTHS to analysis. One subject with hyperuricemia, 2 patients with gout, and one subject with no presence of hyperuricemia or gout disease were selected in this phase. Whole genome of their participants were sequenced by using next generation sequencing. All subjects were matched with gender, and age. The workflow of phase I was summarized in Figure 12.

3.1.2 DNA preparation and whole-genome sequencing.

All DNA was extracted from whole blood by the standard phenol/chloroform method[184]. Red blood cells were lysed using RBC lysis buffer. Subsequently, the blood was centrifuged by 30 microliters of 20 mg/ml proteinase K (Invitrogen, Carlsbad, CA, USA). Three hundred microliters lysis buffer was added for protein denaturation. DNA precipitation was obtained by absolute alcohol. After precipitation, the sample was recovered and separated the DNA pellet by centrifugation.70% ethanol was used to clean the DNA samples. The DNA pellets for each sample were re-suspended by TE buffer. DNA (ng/μL) was measured by a Nanophotometer. The ratio of absorbance at 260 nm and 280 nm was used to assess the optimal and adequate quality of DNA.

Whole-genome sequencing (WGS) was performed at Macogen Inc, Korea (Illumina Inc., San Diego, CA, USA). The sequencing coverage of the whole genome was 15X. Library preparation was prepared on TruSeq PCR-free (350bp) shotgun library prep kit. Sequencing was performed on HiSeq X-ten 150 pair-end. WGS can detect both coding region (exon) and non-coding region. Non-coding region can effect to gene expression such as promotor region, 3'UTR, 5'UTR.

3.1.3 SNPs variants analysis.

All DNAs were sequenced using the Illumina HiSeq X Ten platform (Macrogen, South Korea). Sequence reads were quality checked by FastQC [185] and mapped by the Burrows-Wheeler Alignment tool (BWA version 0.7.17) (http://bio-bwa.sourceforge.net/)(186) using the human reference genome GRCh37 (hg19) downloaded from the UCSC Genome Browser. Variants were called by SAMtools version 1.10 [187] and BCFtools version 1.10.2 [188], followed by the variant annotation by SnpEff version 4.3t [189] using the dbSNP (https://www.ncbi.nlm.nih.gov/snp/), 1000 Genomes (https://www.internationalgenome.org/), ESP6500 (https://evs.gs.washington.edu/EVS/), and ClinVar databases (http://www.ncbi.nlm.nih.gov/clinvar/). The variants were compared and visualized by using the BasePlayer version[190] and IGV version 2.8.2 [191]. The overview of the next-generation sequencing workflow is shown in Figure 13. The definition of sequencing reads, coverage, and variants are shown in Figure 14.

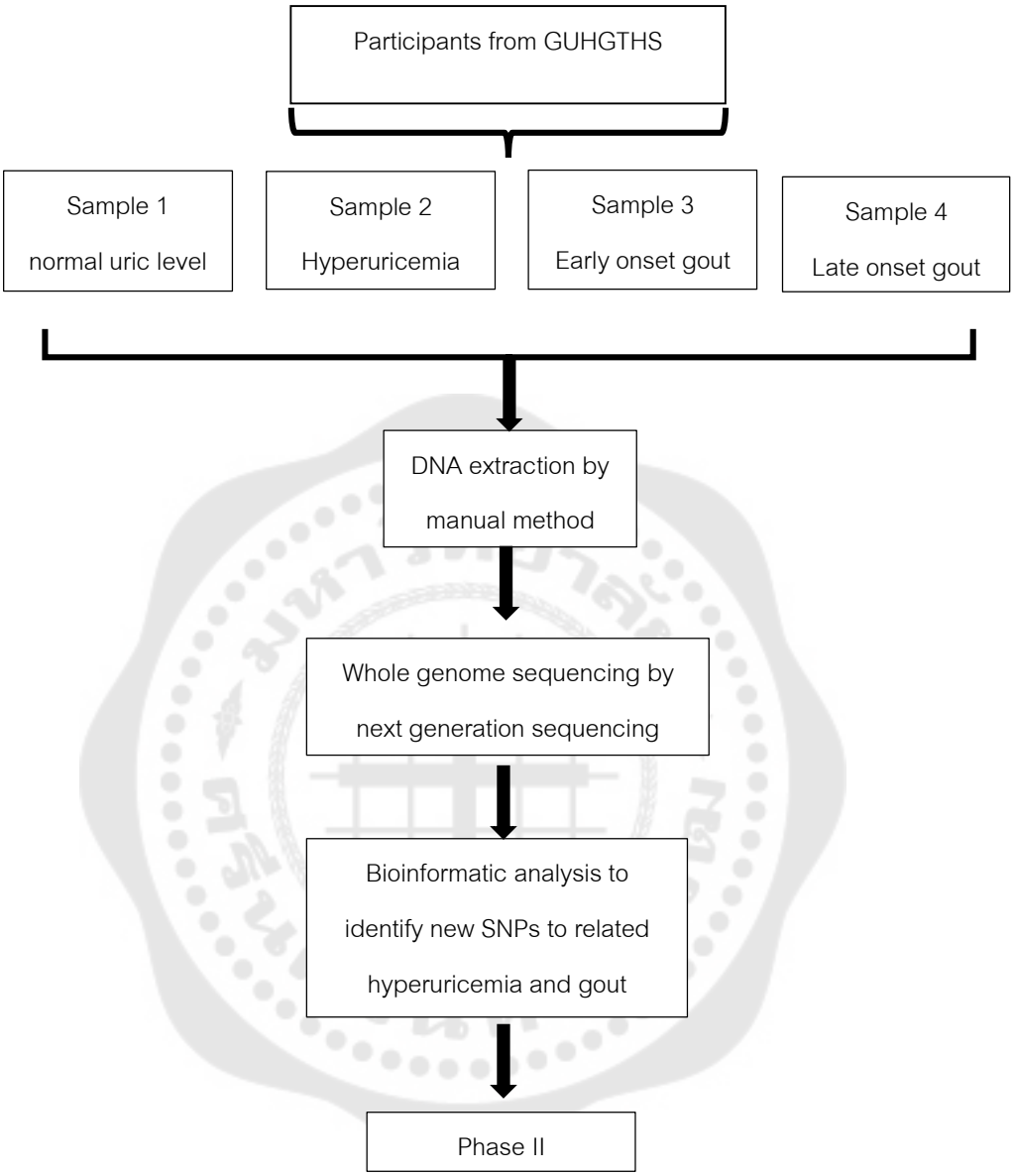

Figure 12 Workflow of the proposed experiment in phase I

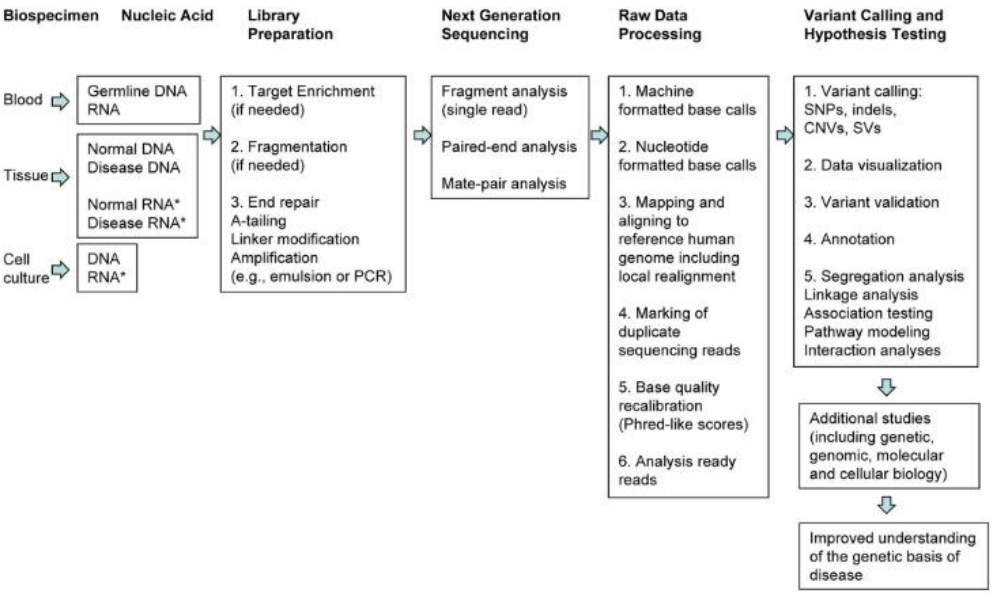

Figure 13 Overview of the next-generation DNA sequencing workflow [176]

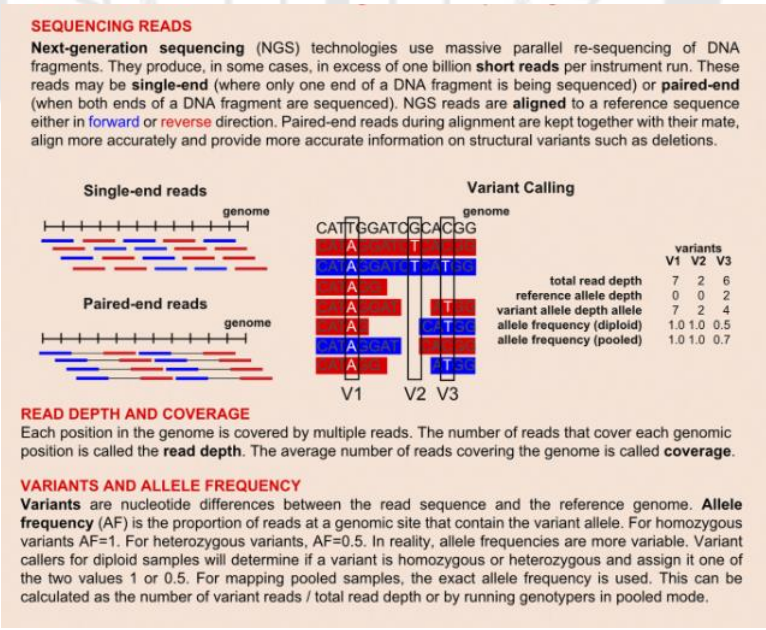

Figure 14 Definition of sequencing reads, coverage, and variants [192]

3.2 Identification of the frequency of LRP2 variants as phase II study

As results from phase I, we found the LRP2 variant (rs2228171), Ala2872Thr (G>A) in hyperuricemia and gout samples. Moreover, we found GLUT9 Ala17Thr only in gout patient. Because LRP2 variant was not present in normal patient so it may be the factor that promote hyperuricemia and gout disease. Changing of nucleotide from G to A at position 2872 resulted in the conversion of alanine to threonine. Missense mutation may lead to changes in protein structure which affects the function and can lead to disorders of uric acid. Of all the above, the objective of phase II was to identify the frequency of LRP2 variants in Thai population. We focused on the patient with non-communicable disease whether we would find the prevalence and relationship of variants in patients with hyperuricemia, heart disease, cerebrovascular disease, hyperlipidemia, hypertension, and obesity (Figure 15). Findings the correlation of genes with diseases provide a greater understanding of pathophysiology. The results can lead to early detection and prevent further disease.

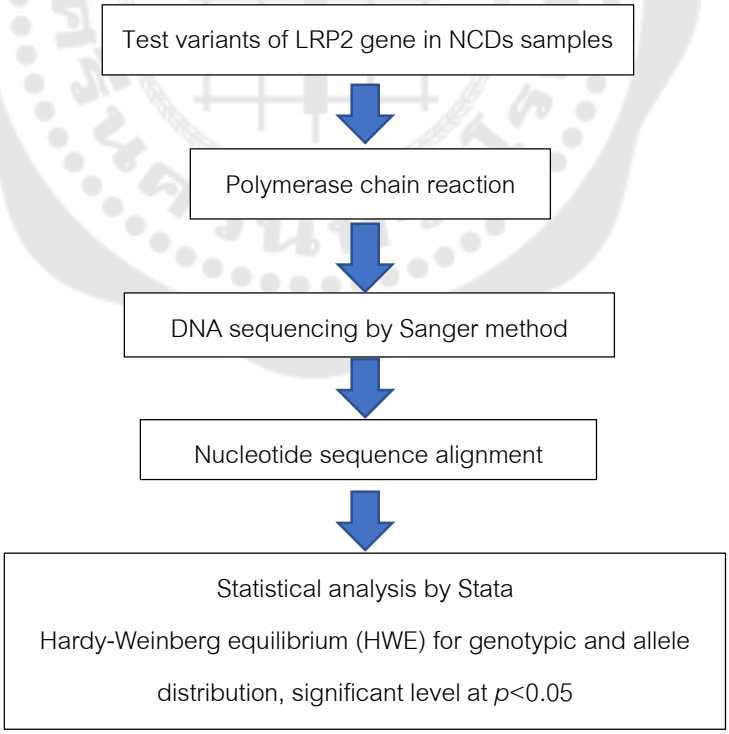

Figure 15 Workflow of the proposed experiment in phase II

3.2.1 Study design, subjects, and data collection

A cross-sectional study was conducted from November 2018 –January 2019. All subjects were selected from patients who diagnosed with non-communicable diseases in Nakhon Nayok hospital, Thailand. All participants had age between 25-60 years. Patients with heart disease (end-stage heart disease, recent myocardial infection within 30 days), stroke (recent stroke within 90 days, bedridden), long term dialysis, cancer, psychiatric disease, pregnancy were excluded from the study. Our study was approved by the review board and Ethics committee of Nakhon Nayok hospital (EC-011/2018). Written inform contented was obtained from all participants.

The sample calculation was used by Cochran (1963). $N=1.96^2 \times P \times (1-P) / d^2$ α=0.05, power (β)=0.80, d=0.2, P=0.11 were calculated in the formula. A total of 550 patients with NCD subjects were recruited in the study (P = prevalence of hyperuricemia in Thai, 11.8 percent)[9].

After screening 550 individuals, we chose 78 whose males with no previous history of gout. Seventy-eight participants had no cancer, no acute heart disease, no receiving uric lowering agents, and on dialysis. Patients with incomplete data were excluded from our study. All participants were given informed consent, reviewed history, collected blood sampling including genotyping. Clinical profiles of our subjects including age, gender, comorbidities, smoking, alcohol use, blood pressure (BP), waist circumference (WC), hip circumference (HC), and body mass index (BMI) were collected. Blood chemistry such as fasting blood sugar (FBS), serum uric acid (SUA), total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), triglyceride (TG), HbA1C, blood urea nitrogen (BUN), creatinine (Cr), and glomerular filtration rate (GFR) were collected from all participants. Genomic DNA was extracted from peripheral blood to find the frequency of variation in patients with hyperuricemia, diabetes mellitus (DM), hyperlipidemia, hypertension (HT), heart disease, and overweight.

3.2.2 Definition of terminology

Hyperuricemia was defined as SUA levels over 7 mg/dL in men, and over 6 mg/dL in women (10). Overweight is classified based on BMI 25-29.99 kg/m^2 by WHO criteria. Diagnosis of DM was described as fasting blood sugar (FBS) equal or greater than 126 mg/dL or 2-hours after glucose tolerance test found plasma glucose equal or more than 200 mg/dL or HbA1C equal or greater than 6.5%[193]. HT was diagnosed follow by systolic BP equal or greater than 140 mmHg or diastolic BP equal or greater than 90 mmHg or antihypertensive medication. Dyslipdemia was defined as cholesterol (over 200 mg/dl), hypertriglyceridemia (over 150 mg/dl), low-density lipoprotein cholesterol (LDL-C over 160 mg/dl) or received lipid-lowering agents. Heart disease was defined as coronary heart disease, ischemic heart disease, hypertensive heart disease, cardiomyopathy excluding congenital heart disease, heart valve diseases, and arrhythmias. Stroke was diagnosed by a physician.

3.2.3 Genetic analysis

3.2.3.1 DNA extraction

The isolation of genomic DNA from peripheral blood was performed using QIAamp DNA Blood Mini kit (QIAGEN, Germany) and stored at -20 C. The DNA concentration and quality were determined using a Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA), a 260/280 ratio of ~1.8, and a 260/230 ratio between 2.0-2.2 were generally accepted.

3.2.3.2 Polymerase chain reaction (PCR)

Forward and reverses primers for rs2228171 were designed by Primer 3 program. Primer sequences of forwarding 5' - 3' and reverse 5' - 3' were TCCATTTTCCAGGCTCAGTC and AGCATCAATCAGCAGCTTCC, respectively. Forward and reverse primers were checked with Primer Blast program. PCR product size was 477 based pair. PCR products were done by PCR Thermal Cycler (Biorad). Then PCR products were run on agarose gel electrophoresis, eluted and purified, respectively.

3.2.3.3 DNA sequencing analysis

DNA sequencing from PCR products was performed using Sanger Sequencing (Macrogen). Chromatogram was analyzed to identify genotypes.

3.2.4 Statistical analysis

All baseline characteristic analyses were performed using STATA version 14 (Stata, College Station, TX). The Hardy-Weinberg equilibrium (HWE) was used to describe genotype and allele distribution. Chi- square test was used to confirm HWE among genotypes ($p<0.05$)[194].

CHAPTER 4
RESULTS

The study results were separated into 2 phases according to the study method as shown below.

4.1 Identification of the novel genetic variation in phase I

The identification of the novel genetic variation related with hyperuricemia and gout was studied in this phase. Two-hundred and fifty participants were enrolled in GUHGTHS. Baseline characteristics according to gender are shown in Table 3.

Table 3 Baseline characteristic of all participants in GUHGTHS *(n=250)*

Characteristics	Gout & HUA (n=125)	Healthy controls (n=125)	*p*-value
Gender (no. Male/Female)	90/35	38/87	
Age (years)	58.99 ± 14.47	47.97 ± 15.23	<0.001
Body mass index (kg/m^2)	26.21 ± 5.26	23.91 ± 3.99	<0.001
Systolic Blood pressure (mmHg)	137.34 ± 18.87	123.83 ± 17.51	<0.001
Diastolic Blood pressure (mmHg)	79.81 ± 13.36	76.78 ± 11.04	0.058
Creatinine (mg/dL)	1.49 ± 1.69	0.87 ± 0.66	<0.001
Total cholesterol (mg/dL)	197.10 ± 48.56	204.90 ± 37.10	0.165
Triglycerides (mg/dL)	154.62 ± 96.98	119.16 ± 60.56	<0.001
HDL-C male (mg/dL)	51.36 ± 24.24	55.27 ± 10.43	0.374
HDL-C female (mg/dL)	59.73 ± 16.92	62.60 ± 13.81	0.373
LDL-C (mg/dL)	112.07 ± 45.30	124.05 ± 37.58	0.039
Fasting blood sugar (mg/dL)	106.57 ± 30.19	99.53 ± 33.91	0.091
Serum uric acid (mg/dL)	6.59 ± 2.03	5.25 ± 0.92	<0.001

HDL-C: high density lipoprotein cholesterol; LDL-C; low density lipoprotein cholesterol Data was presented as mean ± SD and their p-value using independent t-test; gender was presented as p-value using chi-square test

The four male participants including normal uric acid, hyperuricemia, gout, and early onset gout were selected from 193 subjected in the GUHGTHS.They were identified by number A, B, C, and D *(*flow chart as shown in Figure 16*)*.

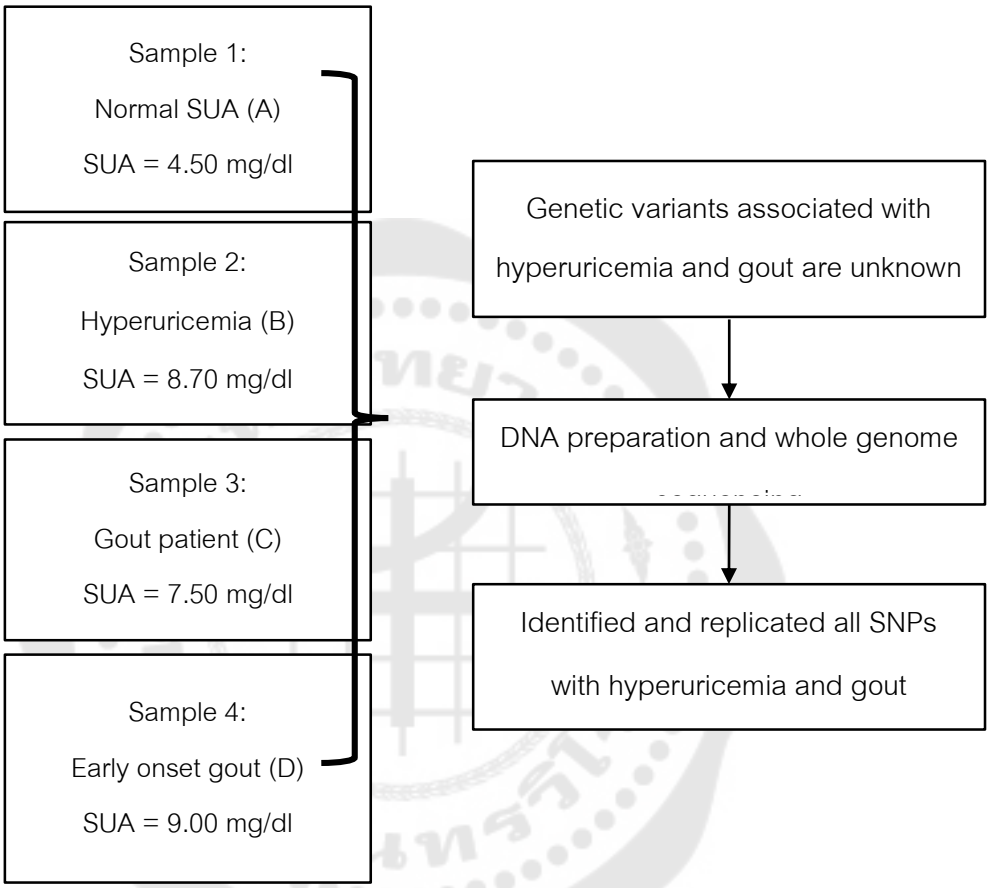

Figure 16 Overview flowchart for investigating variants

Their anthropomorphic and clinical parameters were summarized in Table 4. All 4 participants were male. Subject A, B, and C were mached in age *(*± 5 years*)*. Subject D was not match in age. Subject A and B did not receive drugs or food effecting uric acid level. The extraction of DNA from whole blood was performed, quality checked by nanodrop and gel electrophoresis. Figure 17 showed genomic DNA on gel electrophoresis.

Table 4 Summary of Clinical observations of 4 subjects in phase I of study

ID	Groups	Age(y)	BMI	SBP	DBP	HDL	LDL	TG	TC	FBS
A	Normal	43	22.75	129	85	34	137.0	290	197	344
B	HUA	43	24.83	128	60	55	160.0	66	207	83
C	Gout	37	26.89	121	84	41	137.0	152	209	87
D	Early gout	21	29.40	119	61	39	109.8	166	182	82

BMI: Body mass index, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, TG: Triglyceride,

TC: Total cholesterol, FBS: Fasting blood sugar

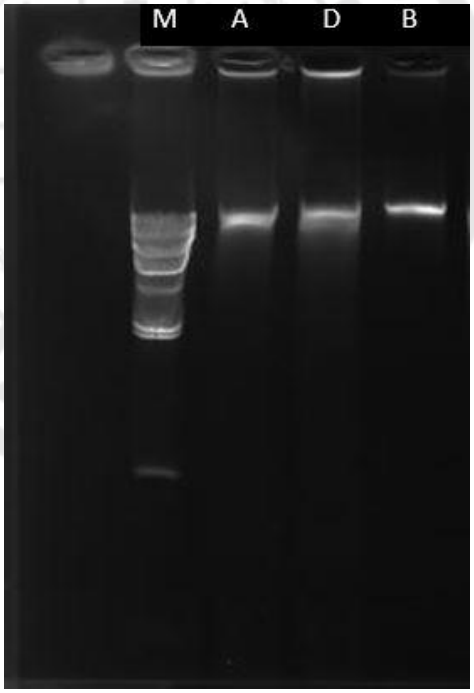

Figure 17 DNA of 3 subjects by 0.8% gel electrophoresis (A, B, D)

A=normal SUA, B=hyperuricemia, D=early onset gout, sample C: DNA was sheared after manual DNA extraction, so whole blood was sent to DNA extraction and genome sequencing at Macrogen (South Korea).

Next generation sequencing analysis was performed on genomic DNA from 4 adult men participants. The data set comprised 118, 599 single-nucleotide variants (SNVs) were selected. Figure 18-21 showed the Genetic variants identified in four participants with hyperuricemia and gout.

We found variants in 17 genes in normal patient (Subject A) such as Adenosine adenase RNA specific (ADAR), Activin A receptor type IIA (*ACVR2A*), Low density lipoprotein receptor-related protein 2 (*LRP2),* Musculoskeletal embryonic nuclear protein 1 (*MUSTN1)*, Soluble carrier family 2 member 9 *(SLC2A9)* or glucose transporter 9 *(GLUT9)*, Transmembrane protein 171(*TMEM171*), Ras responsive element binding protein 1(*RREB1)*, Soluble carrier family 17 member 3 (*SLC17A3*) , Hepatocyte nuclear factor 4 *(HNF4)*, N-acylspingosine amidohydrolase 2 *(ASAH2)*, Soluble carrier family 16 member 9 (*SLC16A9)*, Soluble carrier family 22 member 12 (SLC22A12), Soluble carrier family 5 member 8 (*SLC5A8)*, ATP-binding cassette subfamily C member 4 *(ABCC4)*, Purine nucleoside phosphorylase (*PNP*), Chromosome 17 open reading frame 82 *(C17orf82*) and : soluble carrier family 13 member (*SLC13A3)* as shown in Figure 18.

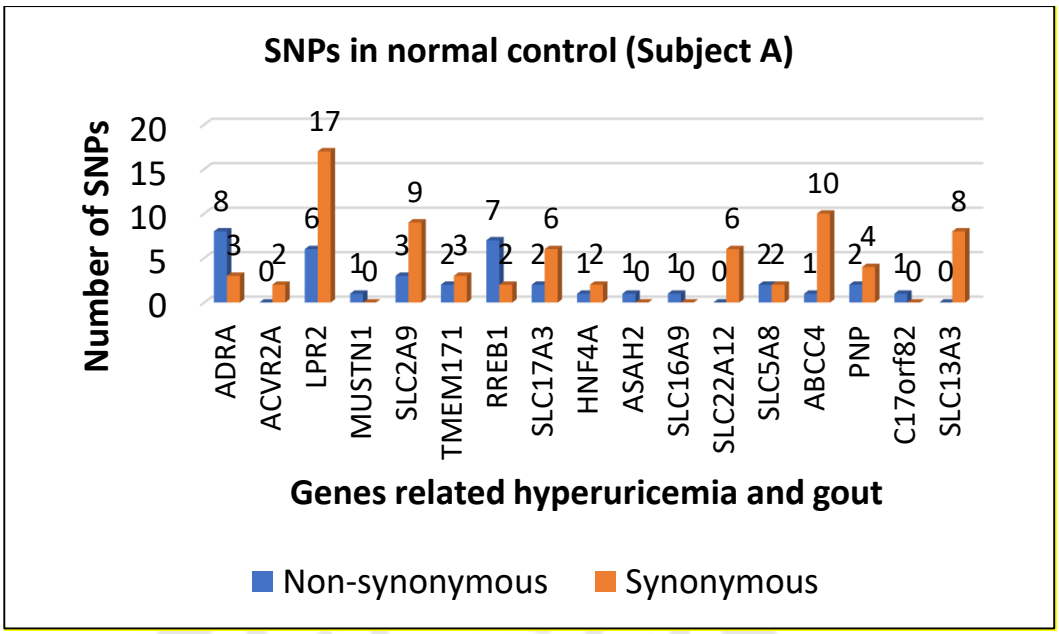

Figure 18 SNPS in normal control (Subject A)

(ADAR: ADAR: Adenosine adenase RNA specific, ACVR2A :Activin A receptor type IIA, LRP2: Low density lipoprotein receptor-related protein 2, MUSTN1: Musculoskeletal embryonic nuclear protein 1, SLC2A9 (GLUT9): glucose transporter 9, TMEM171: Transmembrane protein 171, RREB1: Ras responsive element binding protein 1, SLC17A3: Soluble carrier family 17 member 3, HNF4: Hepatocyte nuclear factor 4, ASAH2: N-acylspingosine amidohydrolase 2, SLC16A9: Soluble carrier family 16 member 9, SLC22A12: soluble carrier family 22 member 12, SLC5A8: soluble carrier family 5 member 8, ABCC4: ATP-binding cassette subfamily C member 4, PNP: Purine nucleoside phosphorylase, C17orf82: Chromosome 17 open reading frame 82, SLC13A3: soluble carrier family 13 member 3)

Variations of 16 genes were found in hyperuricemia (Subject B) such as Adenosine adenase RNA specific *(ADAR)*, Low density lipoprotein receptor-related protein 2 (*LRP2),* Musculoskeletal embryonic nuclear protein 1 (*MUSTN1)*, Soluble carrier family 2 member 9 *(SLC2A9)* or glucose transporter 9 *(GLUT9)*, Transmembrane protein 171(*TMEM171*), Ras responsive element binding protein 1(*RREB1)*, Soluble carrier family 17 member 1 (*SLC17A1*), Soluble carrier family 17 member 3 (*SLC17A3*), N-acylspingosine amidohydrolase 2 *(ASAH2)*, Soluble carrier family 16 member 9

(*SLC16A9*), Soluble carrier family 22 member 12 (*SLC22A12*), Soluble carrier family 5 member 8 (*SLC5A8*), ATP-binding cassette subfamily C member 4 (*ABCC4*), Purine nucleoside phosphorylase (*PNP*), Chromosome 17 open reading frame 82 (*C17orf82*) and Soluble carrier family 13 member (*SLC13A3*) as shown in Figure 19.

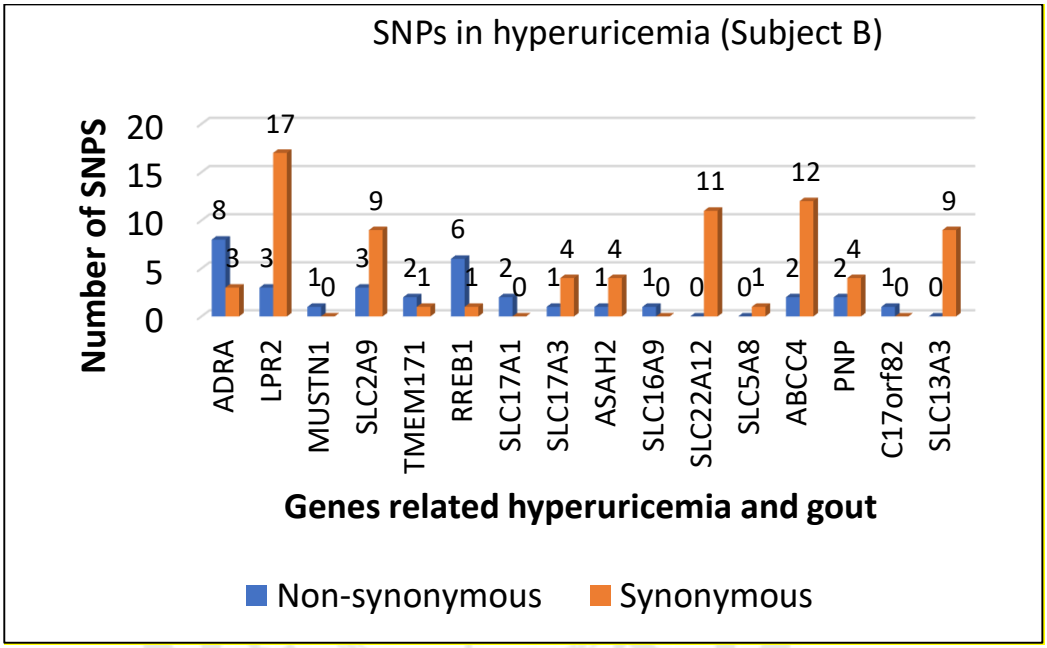

Figure 19 SNPs in hyperuricemia (Subject B)

(*ADAR*: Adenosine adenase RNA specific, *LRP2*: Low density lipoprotein receptor-related protein 2, *MUSTN1*: Musculoskeletal embryonic nuclear protein 1, *SLC2A9* (*GLUT9*): Soluble carrier family 2 member 9 (glucose transporter 9), *TMEM171*: Transmembrane protein 171, *RREB1*: Ras responsive element binding protein 1, *SLC17A1*: Soluble carrier family 17 member 1 , *SLC17A3*: Soluble carrier family 17 member 3, *ASAH2*:: N-acylspingosine amidohydrolase 2, *SLC16A9* : Soluble carrier family 16 member 9, *SLC22A12*: Soluble carrier family 22 member 12, *SLC5A8* : Soluble carrier family 5 member 8, *ABCC4*: ATP-binding cassette subfamily C member 4, *PNP*: Purine nucleoside phosphorylase, *C17orf82*: Chromosome 17 open reading frame 82, *SLC13A3*: Soluble carrier family 13 member)

We found variation of 16 genes in gout patients (Subject C) such as Adenosine adenase RNA specific *(ADAR)*, Activin A receptor type IIA *(ACVR2A)*, Low density lipoprotein receptor-related protein 2 *(LRP2),* Musculoskeletal embryonic nuclear protein 1 (*MUSTN1)*, Soluble carrier family 2 member 9 *(SLC2A9)* or glucose transporter 9 *(GLUT9)*, Transmembrane protein 171 (*TMEM171*), Ras responsive element binding protein 1(*RREB1)*, Soluble carrier family 17 member 1 (*SLC17A1*), Soluble carrier family 17 member 3 (*SLC17A3*), N-acylspingosine amidohydrolase 2 *(ASAH2)*, Soluble carrier family 16 member 9 (*SLC16A9)*, Soluble carrier family 22 member 12 (SLC22A12), Soluble carrier family 22 member 11 *(SLC22A11)*, Soluble carrier family 5 member 8 (*SLC5A8)*, ATP-binding cassette subfamily C member 4 *(ABCC4)*, Chromosome 17 open reading frame 82 *(C17orf82*) and Soluble carrier family 13 member (*SLC13A3)* as shown in Figure 20.

We found variations in 15 genes in early gout patient (Subject D) such as Adenosine adenase RNA specific *(ADAR)*, Activin A receptor type IIA (*ACVR2A*), Low density lipoprotein receptor-related protein 2 (*LRP2),* Musculoskeletal embryonic nuclear protein 1 (*MUSTN1)*, Soluble carrier family 2 member 9 *(SLC2A9)* or glucose transporter 9 *(GLUT9)*, Transmembrane protein 171(*TMEM171*), Ras responsive element binding protein 1(*RREB1)*, Hepatocyte nuclear factor 4 *(HNF4A)*, N- acylspingosine amidohydrolase 2 *(ASAH2)*, Soluble carrier family 16 member 9 (*SLC16A9)*, Soluble carrier family 22 member 12 *(SLC22A12)*, Soluble carrier family 5 member 8 (*SLC5A8)*, ATP-binding cassette subfamily C member 4 *(ABCC4)*, Chromosome 17 open reading frame 82 *(C17orf82*) and Soluble carrier family 13 member (*SLC13A3)* as shown in Figure 21.

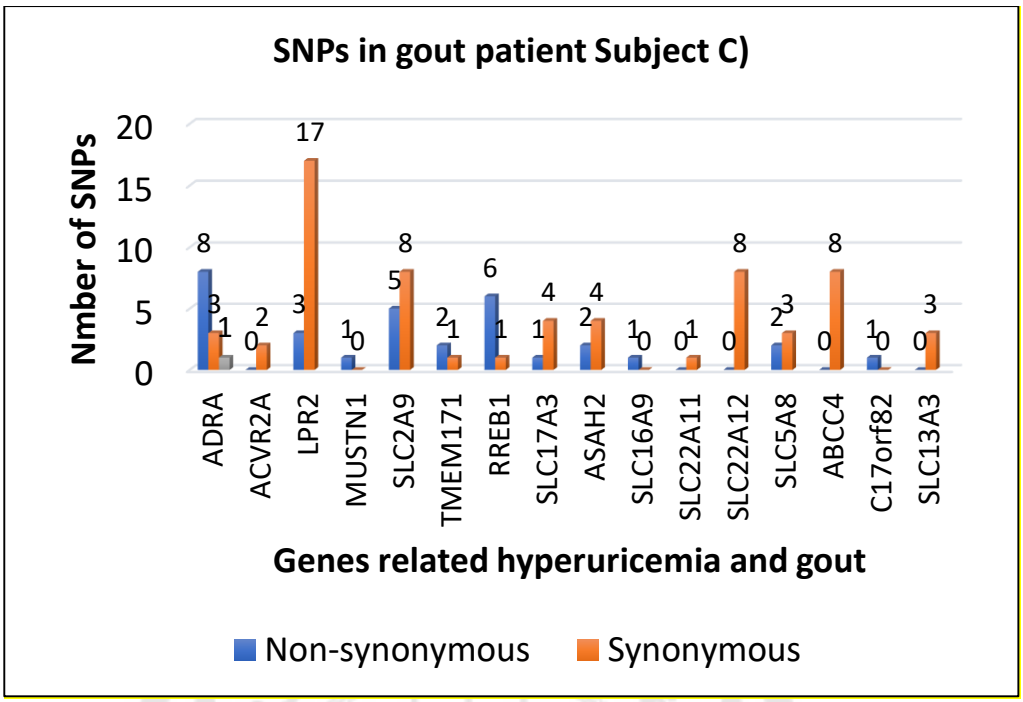

Figure 20 SNPs in gout patient (Subject C)

(*ADAR*: Adenosine adenase RNA specific, *ACVR2A*: Activin A receptor type IIA, LRP2: Low density lipoprotein receptor- related protein 2, *MUSTN1*: Musculoskeletal embryonic nuclear protein 1, *SLC2A9 (GLUT9)* : glucose transporter 9, *TMEM171*: Transmembrane protein 171, *RREB1*: Ras responsive element binding protein 1, *SLC17A3*: Soluble carrier family 17 member 3, *ASAH2*: N-acylspingosine amidohydrolase 2, *SLC16A9*: Soluble carrier family 16 member 9, *SLC22A11*: soluble carrier family 22 member 11 , *SLC22A12*: soluble carrier family 22 member 12, *SLC5A8*: soluble carrier family 5 member 8, *ABCC4*: ATP-binding cassette subfamily C member 4, *C17orf82*: Chromosome 17 open reading frame 82, *SLC13A3*: soluble carrier family 13 member 3)

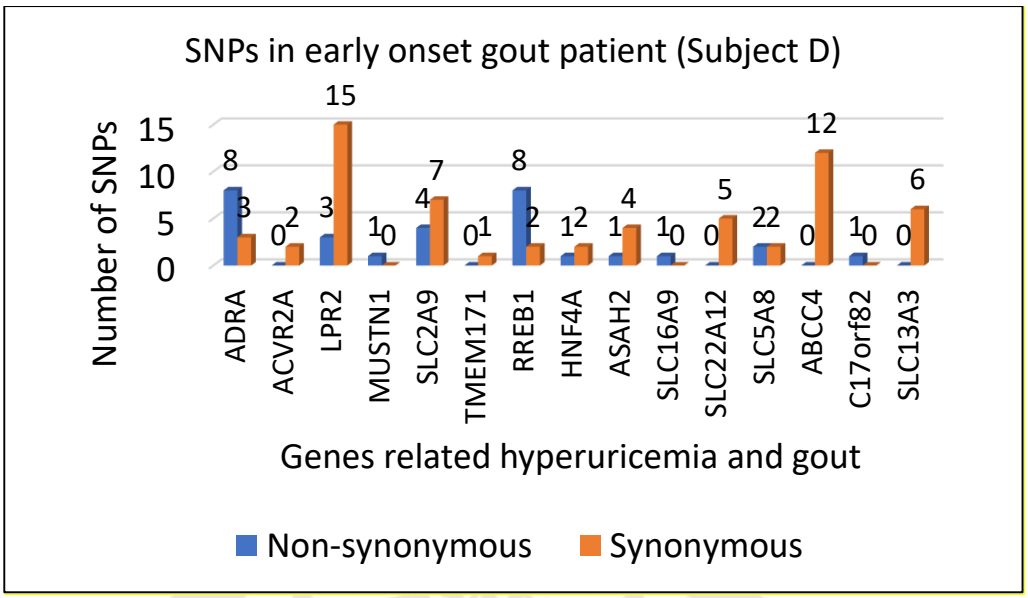

Figure 21 SNPs in gout patient (Subject D)

(*ADAR*: Adenosine adenase RNA specific, *ACVR2A*: Activin A receptor type IIA, *LRP2*: Low density lipoprotein receptor- related protein 2, *MUSTN1*: Musculoskeletal embryonic nuclear protein 1, *SLC2A9 (GLUT9)* : glucose transporter 9, *TMEM171*: Transmembrane protein 171, *RREB1*: Ras responsive element binding protein 1, *HNF4A*: Hepatocyte nuclear factor 4, *ASAH2*: N-acylspingosine amidohydrolase 2, *SLC16A9*: Soluble carrier family 16 member 9, *SLC22A12*: soluble carrier family 22 member 12, *SLC5A8*: soluble carrier family 5 member 8, *ABCC4*: ATP- binding cassette subfamily C member 4, *C17orf82*: Chromosome 17 open reading frame 82, *SLC13A3*: soluble carrier family 13 member 3)

Figure 22 and 23 showed non- synonymous or missense mutation and synonymous in 4 male participants. In our study, we would like to the find the new variant of genes that may be associated with hyperuricemia. So we mentioned on the genes in patient with hyperuricemia (Subject B).

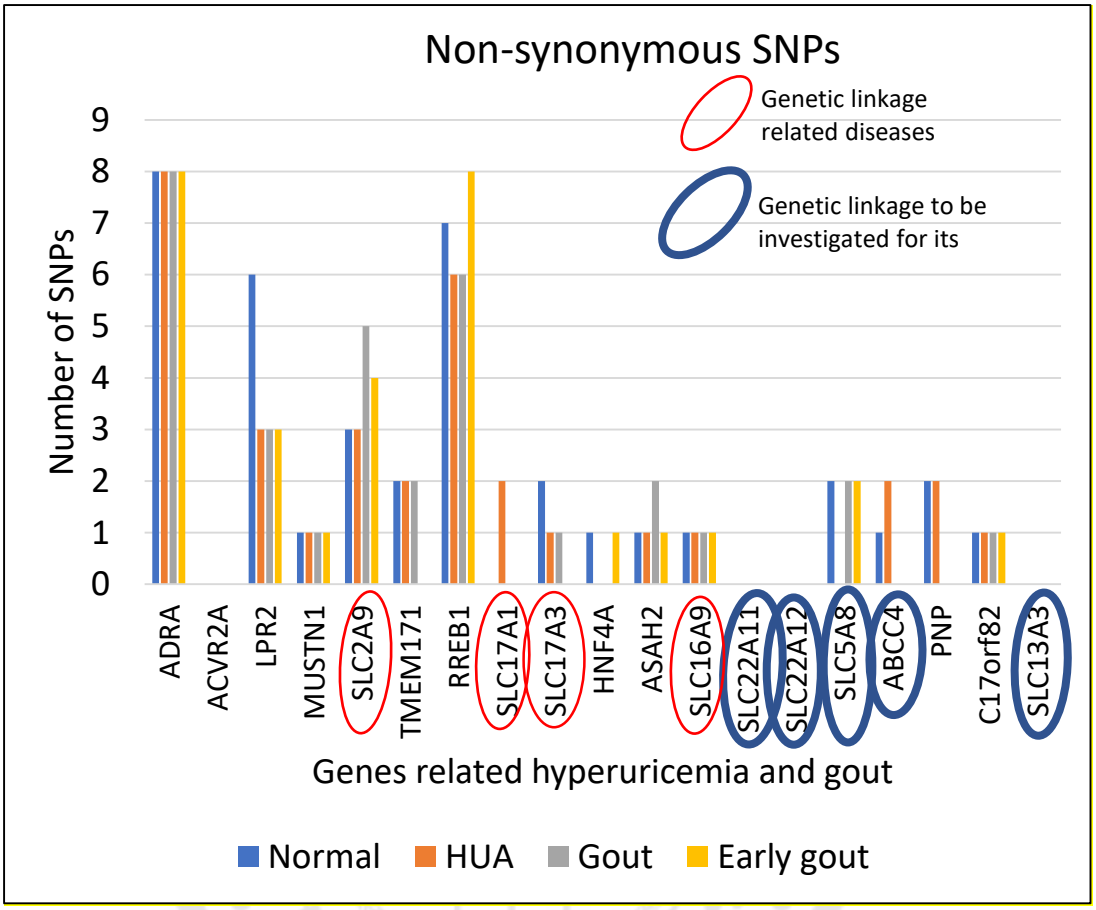

Figure 22 show non-synonymous SNPS in hyperuricemia and gout

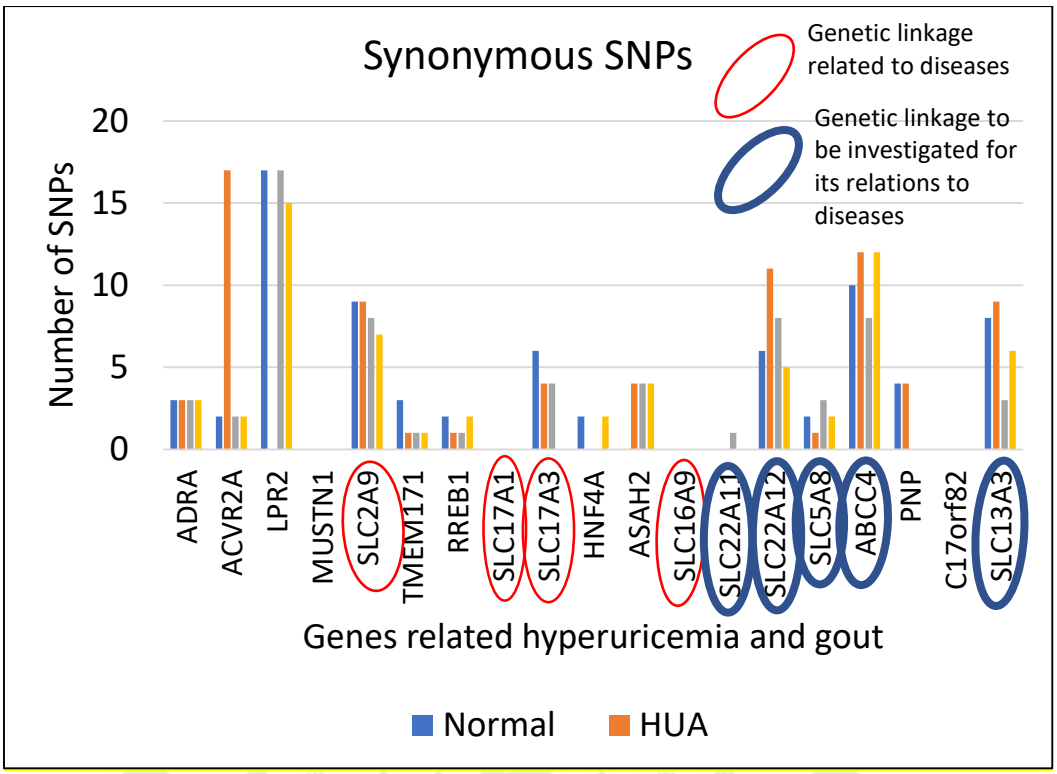

Figure 23 show synonymous SNPs in hyperuricemia and gout

We classified the variants into 4 group by 1) urate transporter gene that evidence to related diseases 2) urate transporter gene which to be investigated for its relation to diseases 3) genes involving in metabolic pathway of urate and 4) unclear of pathway (Figure 24).

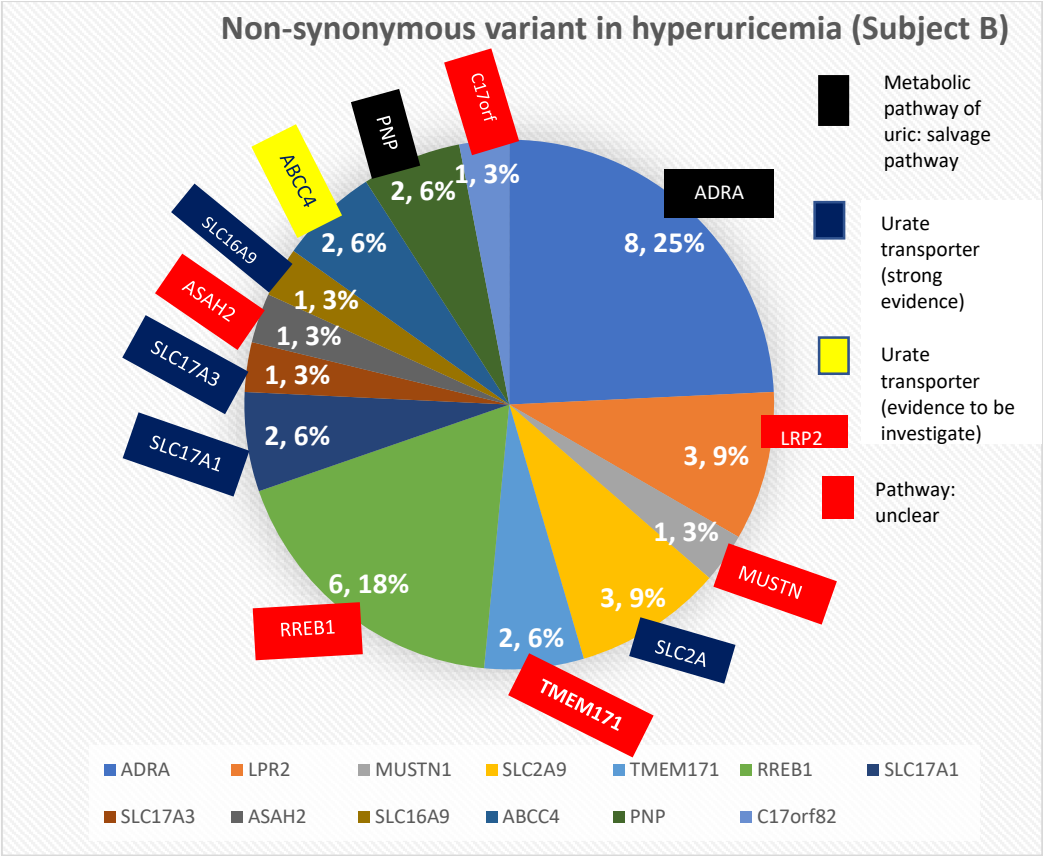

Figure 24 Classification of SNPs in hyperuricemia (Subject B)

When analyzing compare between 4 male participants, genetic variants associated with gout and uric acid were shown in Table 5. The early onset gout subject (Subject D) was identified a missense Ala17Thr, C>T variants in the founder mutation of GLU9, which is a gene located on 4p16.1. We also found a Pro1146Pro synonymous mutation, a known variant of RREB1, in hyperuricemia subject (Subject B), gout patient (Subject C), and early-onset gout subject (Subject D). The Ile223Ile (C>T) mutation of ATP binding protein cassette subfamily C member 4 (ABCCC4) was identified as heterozygous in gout patients (Subject C). However, the Ile223Ile (C>T) mutation was found homozygous in hyperuricemia (Subject B) and early-onset gout subject (ON-652). A missense Ala2872Thr (G>A) variant in LDL receptor-related protein 2 (LRP2) gene was identified in hyperuricemia subjects (Subject B), gout patients (Subject C), and early-onset gout subject (Subject D). The detailed properties of the four mutations on GLUT9, ABCC4, RREB1, and LRP2 are shown in Table 6.

Table 5 Genetic variants found in 4 male participants

Sample ID	Groups	GLU9	ABCC4	RREB1	LRP2
Subject A	Normal	-	-	-	-
Subject B	HUA	-	Ile223Ile	Pro1146Pro	Ala2872Thr
Subject C	Gout	-	Ile223Ile	Pro1146Pro	Ala2872Thr
Subject D	Early gout	Ala17Thr	Ile223Ile	-	Ala2872Thr

GLU9: glucose transporter 9, ABCC4: ATP Binding Cassette Subfamily C Member 4,
RREB1: Ras responsive element binding protein1, Ile: Isoleucine, Pro: Proline,
LRP2: LDL receptor related protein 2, Ala: Alanine, Thr: Threonine

Table 6 Identification of causative and mutations by WGS

Sample ID	Gene	Zygosity	Genomic change	Amino acid change	Location	Mutation type
Subject D	GLU9	Heterozygosity	G49A	Ala17Thr	Chr4 Exon 3	Non-synonymous (missense)
Subject B, Subject D	ABCC4	Homozygosity	C669T	Ile223Ile	Chr13 Exon 6	Synonymous
Subject C	ABCC4	Heterozygosity	C669T	Ile223Ile	Chr13 Exon 6	Synonymous
Subject B, Subject C	RREB1	Heterozygosity	A3438G	Pro1146Pro	Chr 6 Exon 10	Synonymous
Subject B, Subject C, Subject D	LRP2	Heterozygosity	G8614A	Ala2872Thr	Chr 2 Exon46	Non-synonymous (missense)

WGS: whole genome sequencing, Ala: Alanine, Thr: Threonine, Ile: Isoleucine, Pro: Proline,
Chr: Chromosome

4.2 Identify frequency of polymorphism of LRP2 in phase II

Of 78 males from 550 participants was selected to analysis in phase II study. The flow chart of experiment was shown in Figure 25. Baseline characteristic of 78 participants were shown in Table 7. After the isolation of genomic DNA from whole blood, LRP2 rs2228171 was genotyped by PCR *(*Figure 26-28*)*. Genotyping analysis was performed using Sanger sequencing. All participants' chromatograms were shown in appendix A. Pattern of polymorphism was exhibited in Figure 29-31. Data have been described below.

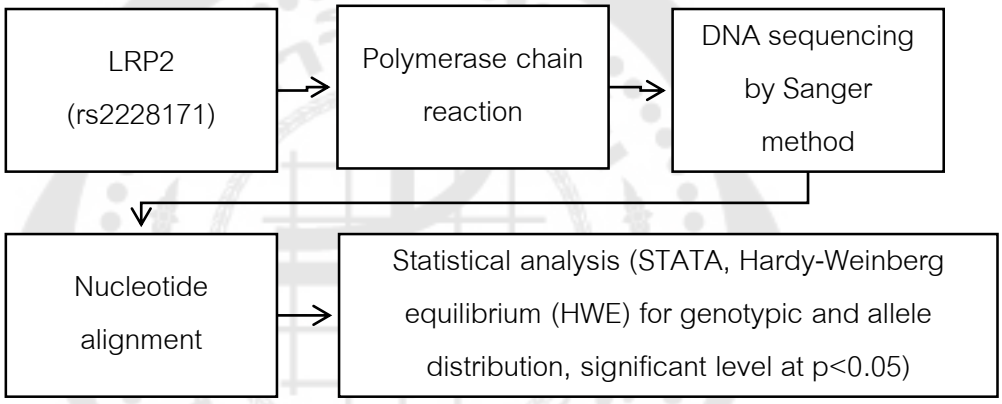

Figure 25 Flow chart of experiment in phase II

4.2.1 Frequency of LRP2 polymorphism and association in hyperuricemia

In our study, we focused on genetic variation in LRP2 rs2228171**.** We clarified allelic frequencies and heterozygosity between hyperuricemia and non-hyperuricemia**.** We categorized into 3 groups for these SNPs 34 homozygous major G-allele carriers, 36 heterozygous carriers, and 8 homozygous minor A allele carrier, rs rs2228171 for G/A**).** We identified no deviation of genotype frequencies from HWE SNP **(**Table 8**).**

Analysis of baseline characteristics between hyperuricemia and non-hyperuricemia group found that no difference between age, smoking status, alcohol status. But BP, TC, TG, LDL, HDL, FBS, BUN, Cr were found more than in the

hyperuricemia group (Table 7). We found no correlation of serum uric acid and genotypes (Table 15). Moreover, there was no association of genotypes and hypeuricemia (Table 16).

4.2.2 Frequency of LRP2 polymorphism and association in diabetes Mellitus

We identified the allelic frequencies and heterozygosity in diabetes mellitus. We found 34, 36, and 8 in homozygous G, heterozygous, and homozygous A in diabetes mellitus, respectively (Table 9). There was no deviation in genotypes between group from HWE SNP.

In data, we found diabetic patients have more than alcohol use, WC, HC, SBP, FBS, HbA1C, BUN, and GFR (Table 7). We found the negative correlation of genotype and serum urate in diabetes mellitus (Table 15). We did not find the relationship of diabetes mellitus and LRP2 genotypes (Table 17).

4.2.3 Frequency of LRP2 polymorphism and association in hypertension

We found 33, 28, and 5 in homozygous G, heterozygous and homozygous A in the HT group, respectively (Table 10). There was a difference in genotypic frequencies from HWE SNP between HT and non-HT (p=0.0099). We found the hypertension group had older age, smoking, alcohol use, HC, WC, TC, TG, LDL, SUA, BUN, Cr greater than the non-HT group (Table 7). We did not find the correlation of hypertension and uric acid (Table 15). However, we found genotype GA associated with hypertension (Table 18).

4.2.4 Frequency of LRP2 polymorphism and association in heart disease

We identified 10 patients with heart disease. There were 2, 7, and 1 patients of homozygous G, heterozygous, and homozygous A (Table 11). There was no difference in genotypic frequencies from HWE SNP between group. We found a greater level of age, WC, HC, TG, LDL, FBS, HbA1C, BUN, Cr in the heart disease group (Table 7). We did not find the correlation of heart disease and serum uric acid (Table 15). Moreover, we did not find the association of genotypes and heart disease (Table 19).

4.2.5 Frequency of LRP2 polymorphism and association in stroke

There were 6, and 1 patient in the stroke group with heterozygous, and homozygous A. We did not find homozygous G in this group (Table 12). Comparison

between stroke and non-stroke patients, we found older age, a greater level of WC, HC, TC, HDL, LDL, SUA, HbA1C, BUN, Cr in stroke group (Table 7). We did not find the correlation of stroke and serum uric acid (Table 15). We could not be analyzed the association of genotypes and stroke due to no genotype GG in stroke patients.

4.2.6 Frequency of LRP2 polymorphism and association in dyslipidemia

We analyzed the variants of LRP2 among the dyslipidemia group. Homozygous major G-allele carriers, heterozygous carriers, and homozygous major AA were found 34, 36, and 8, respectively. We found no difference in genotypic frequencies from HWE SNP (Table 13). We found smoking, alcohol, a higher level of WC, HC, TC, TG, LDL, HbA1C, GFR in the DLP group (Table 7). We did not find the correlation of dyslipidemia and serum uric acid (Table 15). Moreover, we did not find the association of genotypes and dysplidemia (Table 20).

4.2.7 Frequency of LRP2 polymorphism and association in overweight

In our participants, we found half of the patients coexist with overweight. We found 16, 19, and 4 patients with homozygous G, heterozygous, and homozygous A in the overweight group. We found no deviation of genotypic frequencies between groups (Table 14). In patients with overweight, we found frequencies of smoking more than the non-overweight group. WC, HC, SBP, DBP, TG, FBS, and GFR were found in obesity than no obesity (Table 7). However, we did find the correlation of overweight and serum uric acid (Table 15). In Table 21, we did not find the association of genotypes and overweight.

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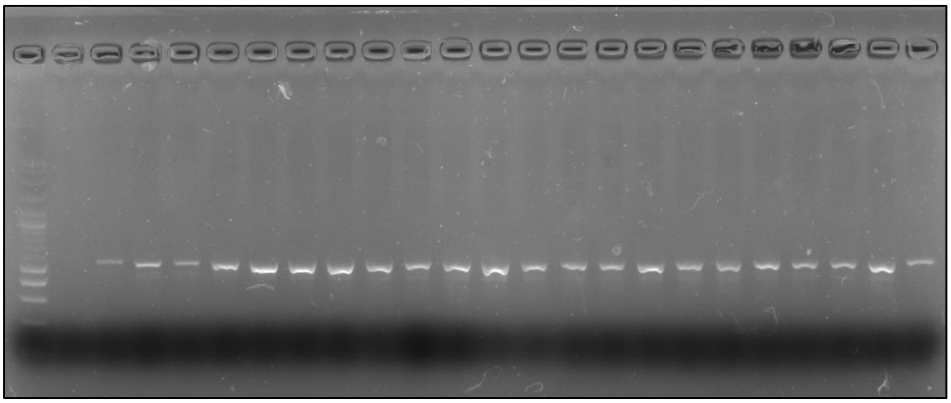
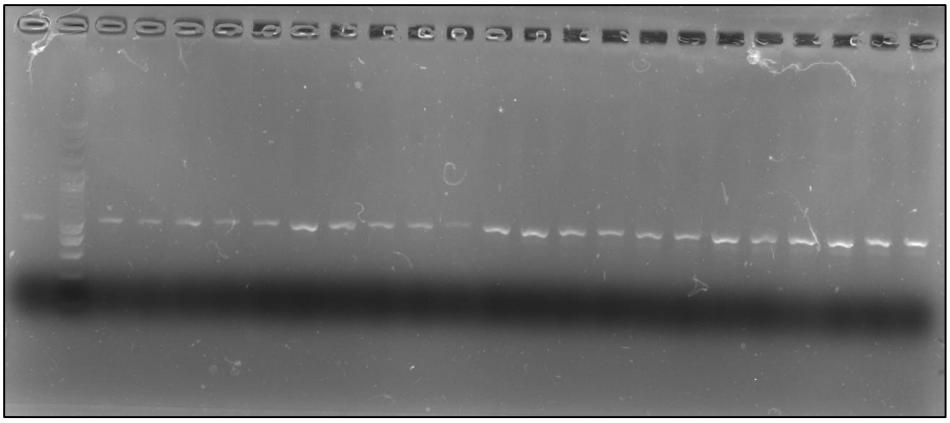
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23



2% agarose gel electrophoresis (product 477 bp)

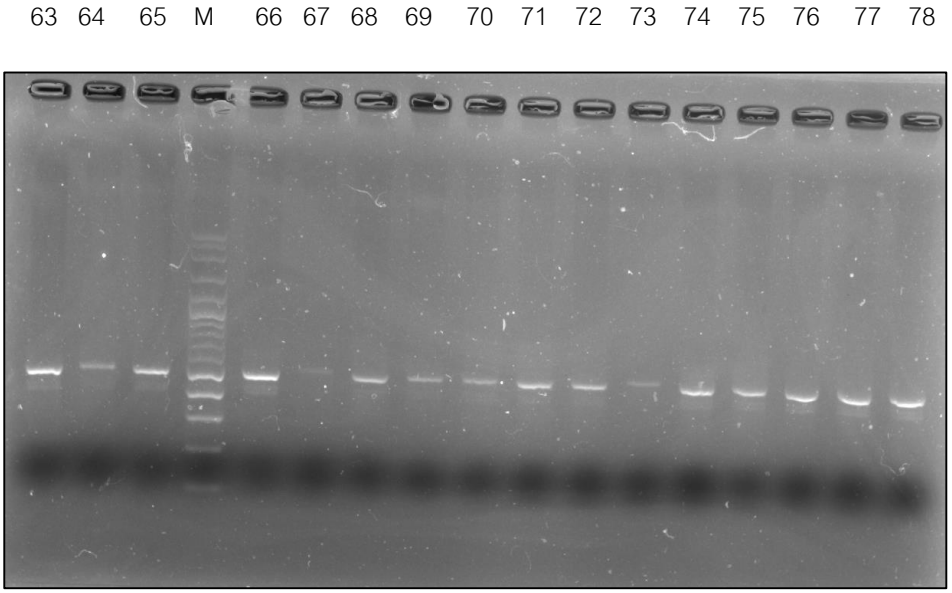
Figure 26 PCR product of LRP2 rs2228171 (samples 1-23)

24 M 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46



2% agarose gel electrophoresis (product 477 bp)

Figure 27 PCR product of LRP2 rs2228171 (samples 24-46)



2% agarose gel electrophoresis (product 477 bp)

Figure 28 PCR product of LRP2 rs2228171 (samples 63-78)

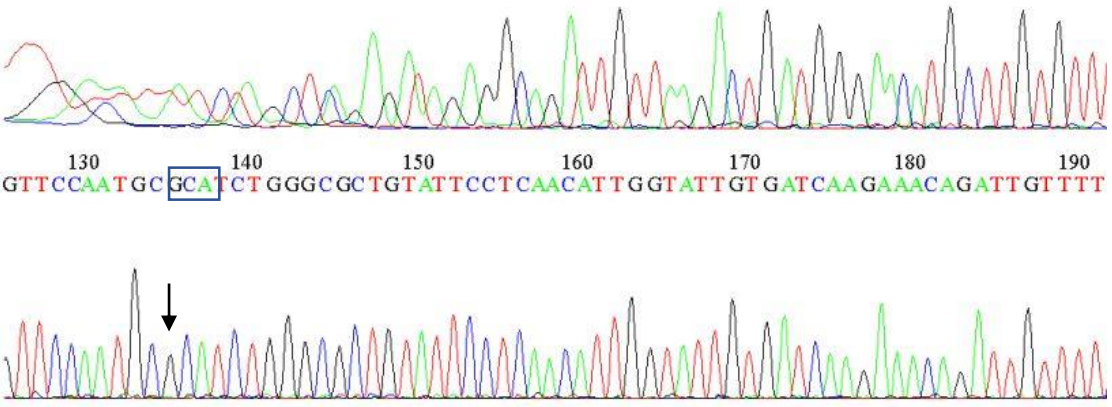


Figure 29 Pattern of chromatogram of LRP2: homozygous G (G/G)

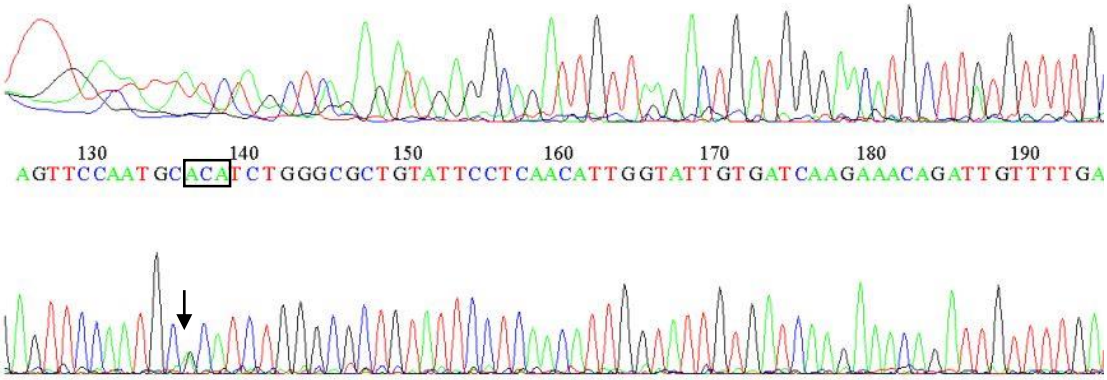


Figure 30 Pattern of chromatogram of LRP2: heterozygous (A/G)

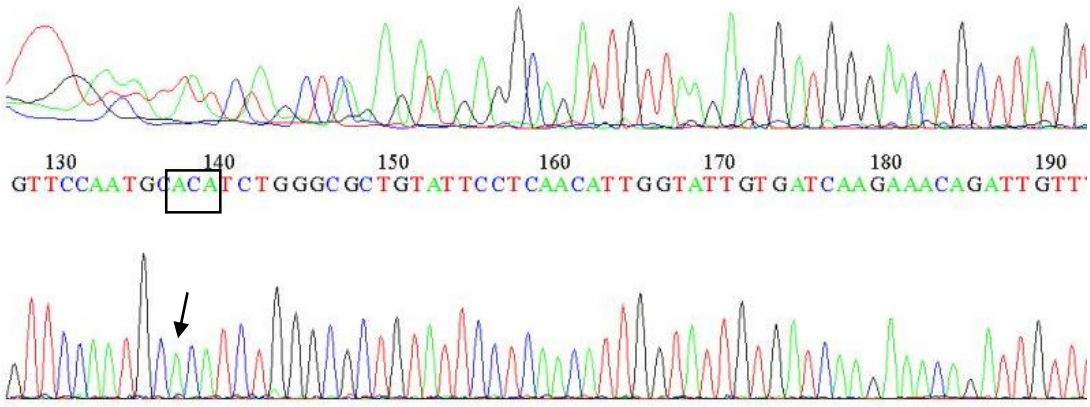


Figure 31 Pattern of chromatogram of LRP2; homozygous A (A/A)

Table 7 Baseline characteristics of all patients in phase II study (N=78)

Factors	HUA/ no HUA	DM/ no DM	HT/ no HT	Heart/ no Heart	CVA/ no CVA	DLP/ no DLP	Overweight/ no overweight
Number	40/38	44/34	66/12	10/68	7/71	53/25	37/41
Age (years)	55.58±9.7/ 55.61±9.92	55.45±9.76/ 55.76±9.76	56.70±9.48/ 49.5±8.94	56.8±7.08/ 55.41±10.06	53±12.52/ 55.85±9.45	55.21±10.18/ 56.4±8.74	53.32±10.46/ 57.63±8.58
Smoking	20/20	21/19	36/4	5/35	5/35	25/15	18/22
History of smoking	10/9	12/7	17/2	3/16	2/17	13/6	11/8
Current smoking	10/11	9/12	19/2	2/19	3/18	12/9	7/14
Alcohol	24/24	27/21	41/7	6/42	4/44	31/17	23/25
History of alcohol	8/4	8/4	12/0	3/9	2/10	8/4	8/4
Current alcohol	16/20	19/17	29/7	3/33	2/34	23/13	15/21
Exercise	25/28	30/23	42/11	7/46	5/48	39/14	22/31

Data are presented as mean +-standard deviation (SD)

Table 7 (continued)

Factors	HUA/ no HUA	DM/ no DM	HT/ no HT	Heart/ no Heart	CVA/ no CVA	DLP/ no DLP	Overweight/ no overweight
WC	90.79±10.65/	92.35±12.53/	90.57±10.97/	94.1±12.64/	93.14±15.56/	91.56±10.82/	98.38±9.60/
(cm)	90.03± 12.33	87.91±9.42	89.58±14.23	89.88±11.24	90.15±11.05	88±12.50	83.23±7.55
HC	97.43±11.43/	99.70±10.22/	98.15±10.41/	102.6±10.24/	100±10.95/	99.21±9.20/	104.51±8.42/
(cm)	98.34±34.21	95.5±10.02	96.33±9.82	97.18±10.18	97.66±10.27	95.04±11.97	91.88±7.87
SBP	135.48±13.47/	133.68±15.23/	135.56±12.96/	131.2±9.94/	128.86±7.49/	132.75±12.96/	135.81±12.59/
(mmHg)	132.98.14.16	135±11.68	127.08±16.13	134.71±14.20	134.79±14.12	137.44±15.00	132.85±14.69
DBP	82.75±10.56/	81.42±10.61/	81.91±9.99/	83.1±11.62/	78±12.65/	78.98±9.96/	81.78±8.25/
(mmHg)	80.03±10.63	82.12±11.88	78.75±13.77	81.18±10.53	81.76±10.43	86.6±10.27	81.10±12.46
TC	179.4±40.79/	167.80±40.48/	177.82±40.30/	163.1±50.11/	179.14±35.44/	179.36±40.3/	178.43±35.24/
(mg/dL)	172.45± 36.91	186.65±34.37	166.08±29.0	177.91±37.00	175.70±39.39	169.92±35.10	178.34±42.15

Data are presented as mean +-standard deviation (SD), WC: waist circumference, HC: Hip circumference

SBP: systolic blood pressure, DBP: diastolic blood pressure, TC: total cholesterol

Table 7 (continued)

Factors	HUA/ no HUA	DM/ no DM	HT/ no HT	Heart/ no Heart	CVA/ no CVA	DLP/ no DLP	Overweight/ no overweight
TG (mg/dL)	158.11±75.51/ 145.79±101.95	143.89±83.64/ 162.74±95.71	155.69±91.06/ 132.42±77.13	180.33±109.88/ 147.96±85.70	137.57±34.88/ 153.54±92.66	160.32±92.49/ 134.68±80.09	162.14±96.70/ 143.05±81.57
HDL (mg/dL)	49.90±21.64/ 48.03±10.29	45.68±10.80/ 52.09±22.29	48.18±17.92/ 50.08±10.82	38.8±12.47/ 49.90±17.16	51.36±19.43/ 48.19±16.85	47.25±13.32/ 51.07±23.02	44.43±9.27/ 52.12±21.18
LDL (mg/dL)	103.20±46.60/ 96.20.36.83	98.79±46.97/ 101.08±35.19	101.67±44.24/ 89.4±25.35	111.11±65.87/ 98.12±37.72	100.26±45.08/ 99.74±42.03	100.85±40.55/ 97.52±45.71	97.16±35.31/ 102.15±47.56
FBS (mg/dL)	152.7±106.98/ 139.82±45.19	174.59±86.75/ 109.97±60.65	138.97±63.39/ 187.42±147.74	159.3±89.10/ 144.53±82.11	106.57±30.19/ 150.35±85.14	145.23±85.90/ 148.96±76.7	156.24±93.27/ 137.56±71.61
SUA (mg/dL)	8.29±0.86/ 5.71±0.91	6.69±1.60/ 7.51±1.40	7.08±1.48/ 6.77±2.01	7.05±1.92/ 7.03±1.52	7.41±1.23/ 7.00±1.60	7.00±1.58/ 7.09±1.56	6.96±1.80/ 7.10±1.33

Data are presented as mean +-standard deviation (SD), TG: triglyceride, HDL: high-density lipoprotein cholesterol, LDL: Low-density lipoprotein cholesterol, FBS: fasting blood sugar, SUA: serum uric acid

Table 7 (Continued)

Factors	HUA/ no HUA	DM/ no DM	HT/ no HT	Heart/ no Heart	CVA/ no CVA	DLP/ no DLP	Overweight/ no overweight
HbA1C	6.86±2.23/ 7.33±1.71	7.35±1.95/ 5.46±0.51	7.05±2.04/ 7.48±1.51	7.91±3.03/ 6.97±1.66	8.65±3.61/ 7.05±1.87	7.45±2.04/ 6.71±1.76	7.18±2.07/ 7.19±1.84
BUN (mg/dL)	15.45±6.40/ 14.03±6.14	15.16±6.27/ 14.24±6.34	14.92±6.57/ 13.83±4.45	19.8±8.02/ 14.01±5.68	15±4.40/ 14.73±6.45	14.49±5.33/ 15.32±8.01	14.70±4.36/ 14.80±7.66
Creatinine (mg/dL)	1.17±0.36/ 1.01±0.26	1.10±0.35/ 1.09±0.28	1.120.21±0.32/ 0.96±0.21	1.31±0.39/ 1.07±0.30	1.17±0.25/ 1.09±0.33	1.09±0.3/ 1.11±0.36	1.11±0.35/ 1.08±0.29
GFR (mL/min/1.73 m^2)	75.63±22.61/ 85.39±18.0	80.55±22.16/ 80.18±19.60	78.11±20.75/ 92.92±18.03	67.6±21.30/ 82.26±20.38	74±17.35/ 81.01±21.27	80.83±20.47/ 79.44±22.33	81.19±21.62/ 79.66±20.57

Data are presented as mean +-standard deviation (SD), BUN: blood urea nitrogen, GFR: Glomerular filtration rate

Table 8 Frequency of LRP2 in hyperuricemia

SNPs	Group	n	Genotype, n(%)			Allele, n (%)		HWE
			G/G	G/A	A/A	G	A	p-value
rs2228171	HUA	40	17 (42.50)	19 (47.50)	4 (10.00)	53 (66.25)	27 (33.75)	0.97
	No HUA	38	17 (44.74)	17 (44.74)	4 (10.52)	51 (67.11)	25 (32.89)	

* Hardy-Weinberg equilibrium test; SNPs: single nucleotide polymorphisms; HUA: hyperuricemia; HUA defined as serum uric acid over 7 mg/dL

Table 9 Frequency of LPR2 in diabetes mellitus

SNPs	Group	n	Genotype, n(%)			Allele, n (%)		HWE
			G/G	G/A	A/A	G	A	p-value
rs2228171	DM	44	20 (45.45)	19 (43.18)	5 (11.36)	59 (67.05)	29 (32.95)	0.82
	No DM	34	14 (41.18)	17 (50.00)	3 (8.82)	45 (66.18)	23 (33.82)	

* Hardy-Weinberg equilibrium test; SNPs: single nucleotide polymorphisms; DM: diabetes mellitus; DM defined as fasting blood sugar more than or equal 126 mg/dL or receiving antidiabetic drug

Table 10 Frequency of LRP2 in hypertension

SNPs	Group	n	Genotype, n(%)			Allele, n (%)		HWE
			G/G	G/A	A/A	G	A	p-value
rs2228171	HT	66	33 (50.00)	28 (42.42)	5 (7.58)	94 (71.21)	38 (28.79)	0.0099
	No HT	12	1 (8.33)	8 (66.67)	3 (25)	10 (41.67)	14 (58.33)	

* Hardy-Weinberg equilibrium test; SNPs: single nucleotide polymorphisms; HT: hypertension; HT was defined SBP over 140 mmHg or DBP over 90 mmHg

Table 11 Frequency of LRP2 in heart disease

SNPs	Group	n	Genotype, n(%)			Allele, n (%)		HWE
			G/G	G/A	A/A	G	A	p-value
rs2228171	Heart	10	2 (20.00)	7 (70.00)	1 (10.00)	11 (55.00)	9 (45.00)	0.22
	No heart	68	32 (47.06)	29 (42.65)	7 (10.29)	93 (68.38)	43 (31.62)	

* Hardy-Weinberg equilibrium test; SNPs: single nucleotide polymorphisms; Heart diseases were diagnosed by physician

Table 12 Frequency of LRP2 in stroke

SNPs	Group	n	Genotype, n(%)			Allele, n (%)		HWE
			G/G	G/A	A/A	G	A	p-value
rs2228171	Stroke	7	0 (0.00)	6 (85.71)	1 (14.29)	6 (42.86)	8 (57.14)	0.013
	No Stroke	71	34 (47.89)	30 (42.25)	7 (9.86)	98 (69.01)	44 (30.99)	

* Hardy-Weinberg equilibrium test; SNPs: single nucleotide polymorphisms; Stroke was diagnosed by physician

Table 13 Frequency of LRP2 in dyslipidemia

SNPs	Group	n	Genotype, n(%)			Allele, n (%)		HWE
			G/G	G/A	A/A	G	A	p-value
rs2228171	DLP	53	23 (43.40)	24 (45.28)	6 (11.32)	70 (66.04)	36 (33.96)	0.9
	No DLP	25	11 (44.00)	12 (48.00)	2 (8.00)	34 (68.00)	16 (32.00)	

* Hardy-Weinberg equilibrium test; SNPs: single nucleotide polymorphisms; DLP: dyslipidemia; DLP defined as total cholesterol over 200 mg/dL, triglyceride over 150 mg/dL, low-density lipoprotein cholesterol over 160 mg/dL or received lipid-lowering agents

Table 14 Frequency of LRP2 in overweight

SNPs	Group	n	Genotype, n(%)			Allele, n (%)		HWE
			G/G	G/A	A/A	G	A	P-value
rs2228171	Overweight	39	16 (41.03)	19 (48.72)	4 (10.26)	51 (65.38)	27 (34.62)	0.89
	No overweight	39	18 (45.15)	17 (43.59)	4 (10.26)	53 (67.95)	25 (32.05)	

* Hardy-Weinberg equilibrium test; SNPs: single nucleotide polymorphisms; Overweight was defined as BMI 25.00-29.99 kg/m^2

Table 15 Correlation of serum uric acid with multiple factors *(n=78)*

Factors	r	95%CI	p
GG	-0.061	-0.280 to 0.164	0.5964
GA	0.058	-0.167 to 0.277	0.0577
AA	0.005	-0.218 to 0.227	0.9675
Hypertension	0.109	-0.116 to 0.324	0.3424
Dyslipidemia	-0.042	-0.262 to 0.182	0.7143
Diabetes mellitus	-0.284	-0.476 to -0.065	0.0118
Heart	0.037	-0.187 to 0.257	0.7502
Stroke	0.088	-0.138 to 0.304	0.4452
Alcohol drinking	-0.006	-0.228 to 0.217	0.9594
Smoking	0.085	-0.140 to 0.302	0.4568
Exercise	-0.099	-0.315 to 0.126	0.3862
Overweight	-0.030	-0.251 to 0.194	0.7966
Systolic blood pressure	0.079	-0.146 to 0.297	0.4903
Diastolic blood pressure	0.025	-0.199 to 0.246	0.8280

GG: homozygous G, GA: heterozygous (G/A), AA: homozygous A, r=correlation

Table 16 Association of genotypes and hyperuricemia

Genotypes	OR (95%CI), p-value	Adjusted OR (95% CI), p-value
GG	0.91 (0.37-2.24), 0.84	1
GA	1.12 (0.46-2.72), 0.81	1.12 (0.44-2.86), 0.816
AA	0.94 (0.22-4.08), 0.94	1.00 (0.21-4.67), 1.00

GG: homozygous G, GA: heterozygous (G/A), AA: homozygous A

Table 17 Association of genotypes and diabetes mellitus

Genotype	OR (95%CI), p-value	Adjusted OR (95% CI), p-value
GG	1.19 (0.48-2.94), 0.706	1
GA	0.76 (0.31-1.87), 0.55	0.96 (0.35-2.60), 0.930
AA	1.32 (0.29-5.98), 0.715	1.43 (0.25-8.29), 0.687

GG: homozygous G, GA: heterozygous (G/A), AA: homozygous A

Table 18 Association of genotypes and hypertension

Genotype	OR (95%CI), p-value	Adjusted OR (95% CI), p-value
GG	11.0 (1.34-90.12), 0.025	1.00
GA	0.37 (0.10-1.35), 0.131	0.11 (0.012-0.90), 0.040
AA	0.25 (0.05-1.21), 0.084	0.05 (0.004-0.59), 0.017

GG: homozygous G, GA: heterozygous (G/A), AA: homozygous A

Table 19 Association of genotypes and heart disease

Genotype	OR (95%CI), p-value	Adjusted OR (95% CI), p-value
GG	0.28 (0.06-1.42), 0.125	1
GA	3.14 (0.75-13.18), 0.118	3.86 (0.74-20.11), 0.108
AA	0.97 (0.11-8.82), 0.977	2.29 (0.18-28.86), 0.523

GG: homozygous G, GA: heterozygous (G/A), AA: homozygous A

Table 20 Association of genotypes and dyslipidemia

Genotype	OR (95%CI), p-value	Adjusted OR (95% CI), p-value
GG	0.98 (0.37-2.54), 0.960	1
GA	0.90 (0.35-2.33), 0.822	0.96 (0.35-2.60), 0.930
AA	1.47 (0.27-7.85), 0.653	1.43 (0.25-8.29), 0.687

GG: homozygous G, GA: heterozygous (G/A), AA: homozygous A

Table 21 Association of genotypes and overweight

Genotype	OR (95%CI), p-value	Adjusted OR (95% CI), p-value
GG	0.79 (0.32-1.94), 0.606	1
GA	1.21 (0.50-2.95), 0.675	1.27 (0.49-3.25), 0.622
AA	1.12 (0.26-4.84), 0.878	1.27 (0.27-5.92), 0.764

GG: homozygous G, GA: heterozygous (G/A), AA: homozygous A

CHAPTER 5
CONCLUSION AND DISCUSSION

The study conclusions and discussion were separated into 2 phases according to the study method as shown below.

5.1 Conclusion and discussion in phase I

In phase I study, we have found the non-synonymous SNPs in the Glucose transporter 9 (GLUT-9)[56, 115-118, 138, 139, 141-143], Soluble carrier family 17 member 1 (SLC17A1) or NPT1 [56, 118, 138, 140, 143-146], and Soluble carrier family 17 member 3 (SLC17A3) or NPT4 [56, 140, 147] that were previously reported to be associated with hyperuricemia and gout. Moreover, we have found the non-synonymous SNPs which previously report associated with serum uric acid such as Soluble carrier family 16 member 9 (SLC16A9)[56], Low density lipoprotein receptor related protein 2 (LRP2) [115], Musculoskeletal embryonic nuclear protein 1 (MUSTN1)[56], Transmembrane protein 171 (TMEM171)[56], Chromosome 17 open reading frame 82 (C17orf82)[56], Ras responsive element binding protein 1 (RREB1) [56], and N-acylspingosine amidohydrolase 2 (ASAH2) [56] in patient with hyperuricemia.

After comparison of 4 participants (normal uric acid, hyperuricemia, gout, and early onset gout) we have found the missense Ala17Thr (G>A) glucose transporter 9 (GLUT9) mutation in only early-onset gout. We identified the synonymous Pro1146Pro, A>G ras responsive element binding protein1 (RREB1) mutation in hyperuricemia and gout. Whole-genome sequencing also identified Ile223Ile (C>T) ATP Binding Cassette Subfamily C Member 4 (ABCC4) mutation and Ala2872Thr (G>A) LRP2 mutation as the genetic finding in patients with hyperuricemia, early-onset gout, and gout, respectively.

SLC2A9 gene encodes soluble carrier family 2 member 9, also named glucose transporter 9 (GLUT9). GLUT 9 is a major role in urate homeostasis. GLUT 9 is a reabsorptive urate transporter. Multiple common variant of GLUT9 have been linked to increased risk of development of hyperuricemia, renal overload gout, renal under-excretion gout, and overall risk of gout. But the low-frequency variants associated with

renal hypouricemia type 2[56, 115-118, 138, 139, 141-143]. GLUT9 variant also identified in early-onset gout in our study similarly to previous reports.

LRP2 gene encodes low-density lipoprotein receptor-related protein 2 (megalin), it is a member of the low-density lipoprotein receptor (LDLR) family. The expression of Megalin is found on epithelial of renal proximal tubules and endocytosis[195]. LRP2 is a glycoprotein, its molecular weight is about 600 KD. The structure of LRP2 consists of a large amino-terminal extracellular domain which has a one transmembrane domain and a carboxy-terminal cytoplasmic tail. The extracellular domain has four clusters (I-IV) of LDLR class A that replicates on ligand-binding regions. Ligands of Megalin include apolipoproteins B, E, and lipoprotein lipase [196, 197]. Several study reported positive and negative correlation of LRP2 gene associated with hyperuricemia [132, 139, 198-200]. Recently, the study showed an intron variant of LRP2 rs2544390 on chromosome 2q24-31 that is associated with hyperuricemia[139]. Study from Japanese population found the association of LRP2 gene rs2544390 with body mass index and urate level [201]. Previous reported the LRP2 polymorphism associated with plasma lipid levels[202]. Several study reported positive and negative correlation of LRP2 gene associated with hyperuricemia [132, 139, 198-200].

Our previous study in phase I have found the polymorphism of LRP2 Ala2872Thr (rs2228171) in patients with hyperuricemia and gout. LRP2 expressed on the glomeruli and proximal tubular cells of kidney[203]. So, LRP2 may be affect to urate hemostasis. For this reason, we continued study in phase II to identifiy the association between rs 2228171 and hyperuricemia.

5.2 Conclusion and discussion in phase II

Previous phase I study "GUHGTHS" identified that one novel locus mapped to LRP2 (rs2228171) (Ala2872Thr, G>A) has been found in hyperuricemia, and gout in Thai men, but a small number of a participant. So, we need to evaluate the LRP2 gene in a large population. Our study was designed to identify the frequency of the LRP2 gene

(rs2228171) in hyperuricemia, heart disease, cerebrovascular disease, hyperlipidemia, hypertension, and obesity among Thai NCD patients.

LRP2 is the member of the low-density lipoprotein (LDL) receptor family. LRP2/Megalin expressed on the apical surface of absorptive epithelial tissues. It is mainly in the kidney, particulary in glomeruli and proximal tubular cells [195]. Interestingly, the previous study demonstrated that the LRP2 protein is important for reuptake of numerous ligands such as lipoproteins, nutrients, morphogens, protease-protease inhibitor complexes including vitamin-vitamin binding protein complexes [195, 204]. These proteins also have a role in cell signaling. Mutation of LRP2/Megalin can lead to Donnai-Barrow syndrome (DBS) and facio-oculoacoustico-renal syndrome (FOAR). Megalin is still expressed on thyrocytes, it is a receptor for thyroglobulin[203]. Megalin mediates transcytosis of thyroglobulin (Tg), thyroid hormone precursor[205]. Previous study in mice with megalin deficiency found thyroid dysfunction[206].

5.2.1 LRP2 gene associated with hyperuricemia, and gout

LRP2 could be associated with serum uric acid through urate-binding proteins endocytosis. In our study, we have not been found significant genotypic frequencies between SNP in the gene encoding LRP2 and hyperuricemia among Thai NCD patients. Some previous GWAS in a Japanese population identified common novel variant in LRP2 (rs2544390) associating with serum uric acid [139]. However, GWAS in a Chinese population revealed no significant association of rs2544390 and urate [132]. Moreover, study in Japanese population reported the relationship of rs2544390 and body mass index including serum uric acid [201]. Study in Chinese population [198] and cohort study in Māori and Pacific Island [199] also showed rs2543390 influencing on gout susceptibility. However, the protective effect was identified in European study[199] and no association of gout susceptibility was reported in a Japanese population [200].

However, megalin medidates transcytosis of thyroglobulin[205]. It is known that thyroid functions in the metabolism, growth and development of human body. Variant of LRP2 may be induced to thyroid dysfunction. We propose that polymorphism of LRP2 may be additional factor to metabolism that controls urate homeostasis.

Because positive and negative results of LRP2 were reported. Therefore, we cannot be concluded that having variant increased the risk of hyperuricemia. Although the position of LPR2 in our study is different from the Japanese and Chinese populations. But this is a novel position that has been found in Thai people. Even the results were not related to hyperuricemia. New information on this gene is found.

5.2.2 LRP2 gene associated with DM and hyperlipidemia

LRP2 mediated endocytosis of LDL. LRP2 is a lipoprotein receptor that play role in cholesterol transport [207], use with its coreceptor cubilin [208] Moreover, megalin is a receptor for apoJ/clusterin that involve HDL particles [209, 210] and Lp (1), Lp (1) is an atherogenic particle [211, 212]. Apolipoprotein M is found in pre-β-HDL particles, chylomicrons, LDLs and VLDLs. Liver and kidney secreted Apolipoprotein M, and megaling receptor involve in this secretion [213] [214, 215]. Cubilin is a coreceptor of Megalin. ApoA1-I and ApoA-II are structure of HDL that need Cubilin and Megalin [216] [217]. Thus megalin involves with the regulation of HDL metabolism. A report from the study in the Japanese population found a level of cholesterol-related to genetic variation in the megalin gene [202].

Dyslipidemia can increase insulin resistance and serum uric acid levels (218). A report from the Japanese population showed LRP2 (rs2229268) variants related with LDL and serum uric acid level in humans [202]. Although, we did not find the association of genotypes with dyslipidemia and diabetes mellitus. The fact that we cannot find the association may be due to the difference in positions of the LRP2, ethnic diversity, and the number of population studies is relatively small. Therefore, further investigation is required.

5.2.3 LRP2 gene associated with hypertension

Megalin and its ligands are involved in some disease in kidney and brain [203]. Ligands of Megalin including PTH, albumin, insulin, leptin, and angiotensin II need megalin receptor to endocytosis and bring the ligand into lysosome. Previous report showed the rold of Megalin associated with disease including hypertension, diabetes, renal and obesity [219-225]. In our study, we found the patients with homozygous A had

protective effect in HT. So variants of the LPR2 gene might be protect hypertension. However, since the majority of the population studied coexisted with HT. There were few patients without HT. Therefore, we cannot conclude whether homozygous A presence decrease s the risk of HT. The authors suggest further study is required.

5.2.4 LRP2 gene associated with stroke and heart disease

Several risk factors such as dyslipidemia, hypertension, and diabetes mellitus increase risk of stroke and heart disease. Variants of LRP2 may promote DM, obesity, HT, and DLP [219-222] [223] [224, 225], which leading to stroke. We cannot define the association of genotypic, stroke, and heart diseae due to small smple size. We need to investigate and design the study to find the risk of genotype in stroke disease.

In conclusion, our study is the first cross-sectional study of the frequency of the rs variants in the LRP2 rs2228171 gene in Thai NCDs patients. We identified the genetic frequencies of LRP2 rs2228171 with hyperuricemia and DM, hyperlipidemia, heart, stroke, HT, and obesity. Although we did not find the association of genotypes in hyperuricemia. But our results provide the association of LRP2 genotypes in HT patients. Furthermore, the study will be done to clarify the effect of LRP2 variation and metabolic diseases such as HT. The future research will lead to a better understanding of the association of LRP2.

Appendix

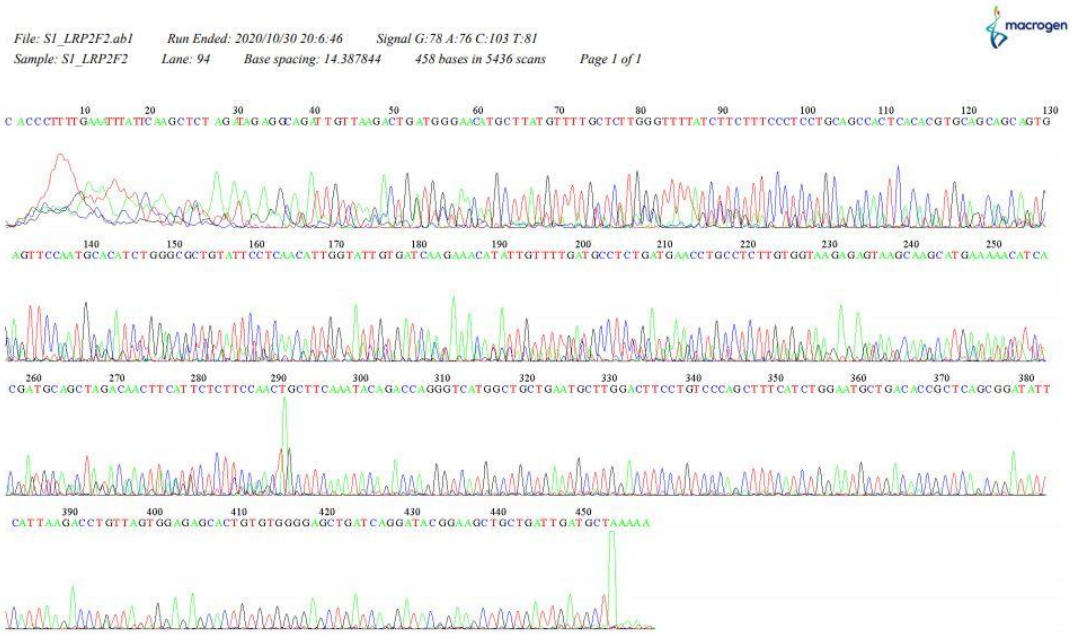


Figure 32 Chromatogram of sequencing from PCR-LRP2 sample 1

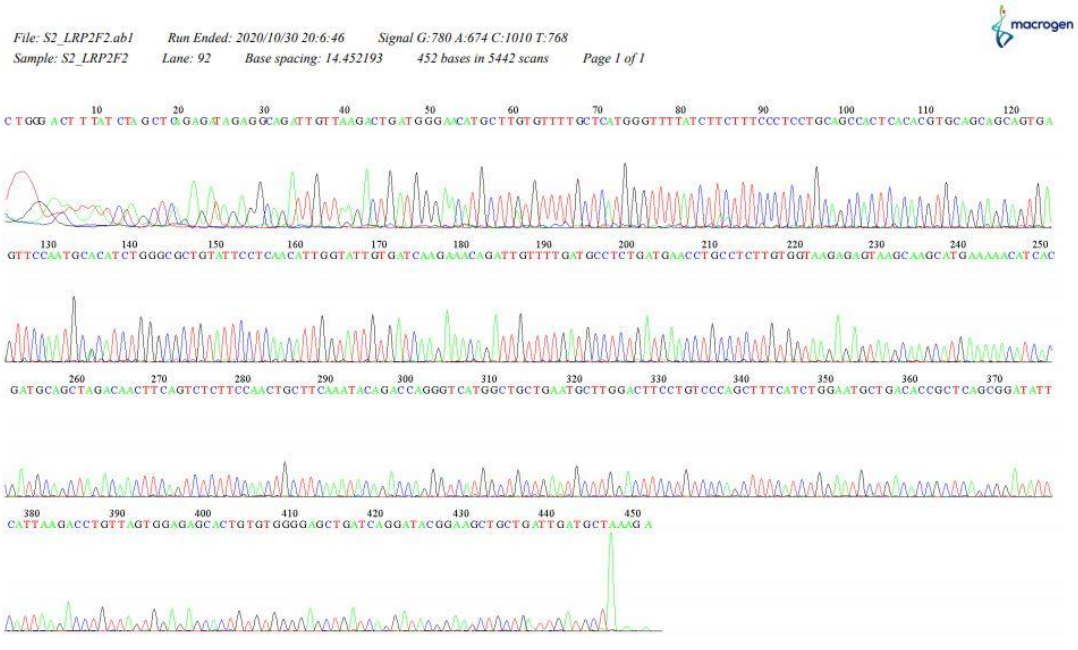


Figure 33 Chromatogram of sequencing from PCR-LRP2 sample 2

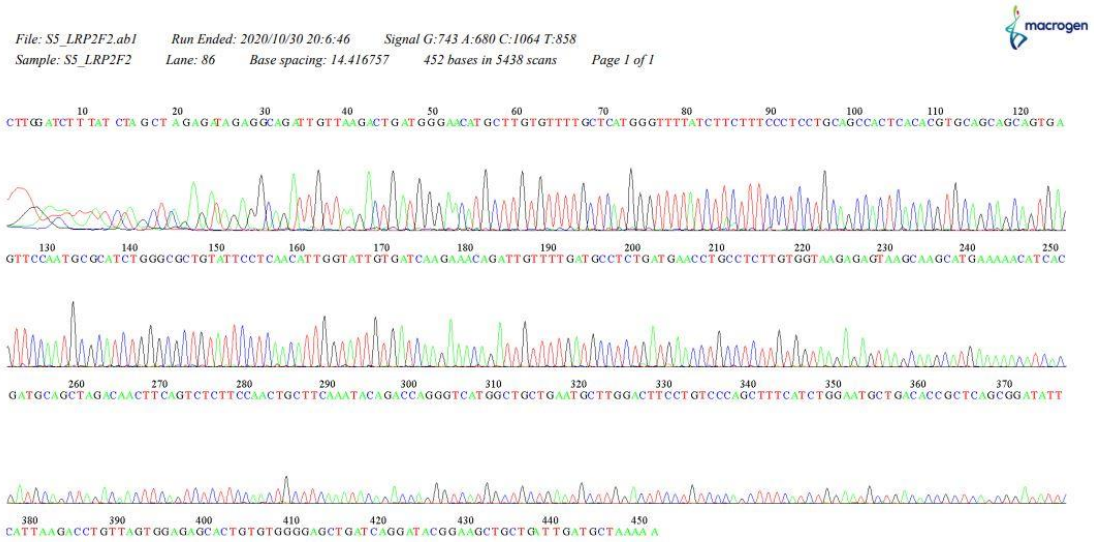


Figure 34 Chromatogram of sequencing from PCR-LRP2 sample 3

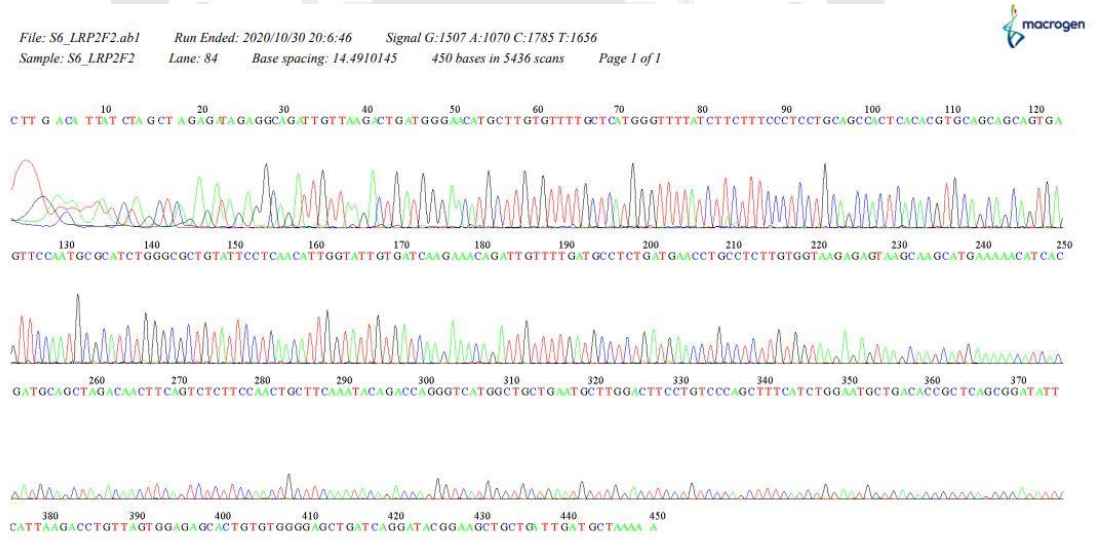


Figure 35 Chromatogram of sequencing from PCR-LRP2 sample 4

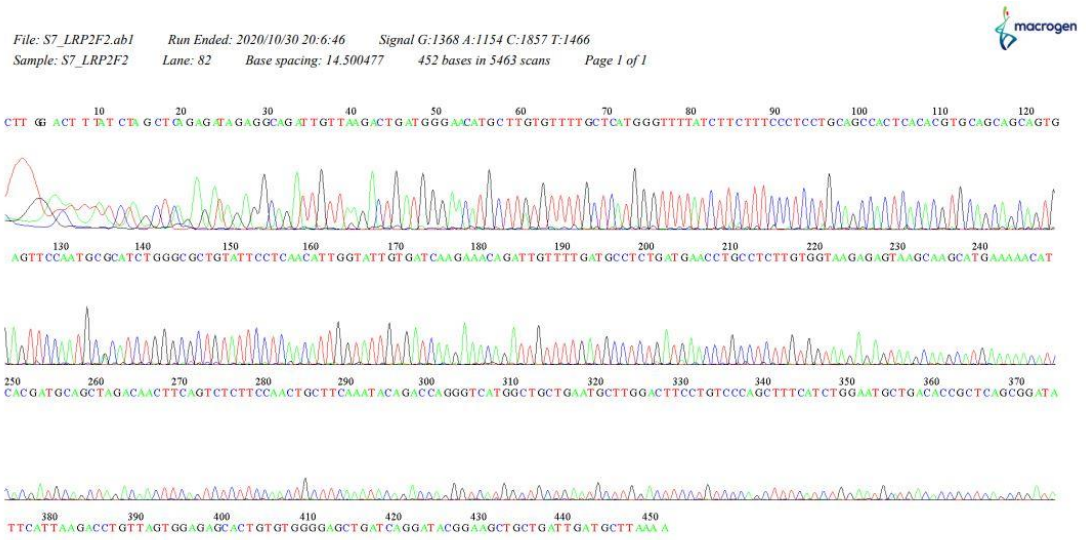


Figure 36 Chromatogram of sequencing from PCR-LRP2 sample 5

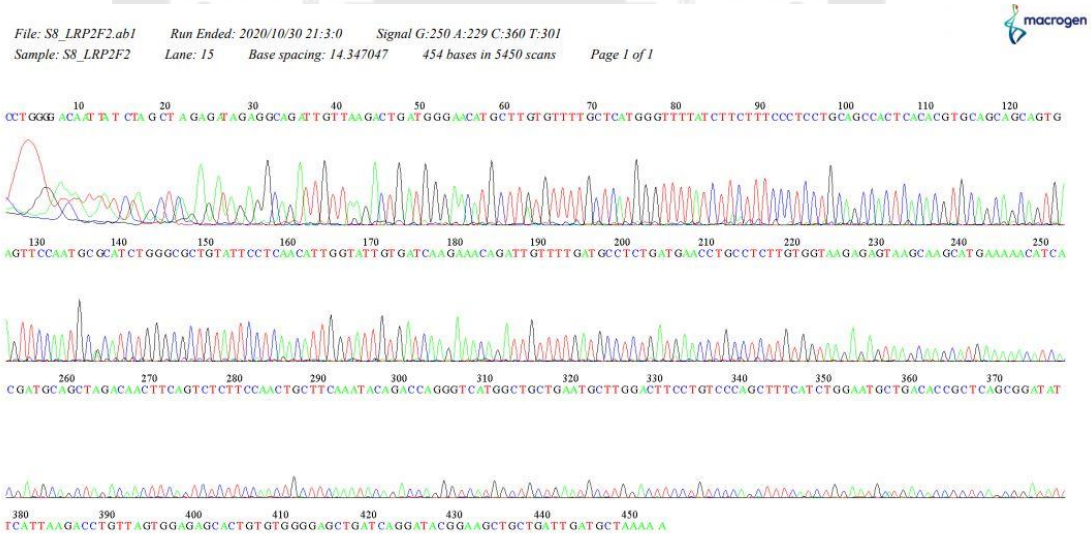


Figure 37 Chromatogram of sequencing from PCR-LRP2 sample 6

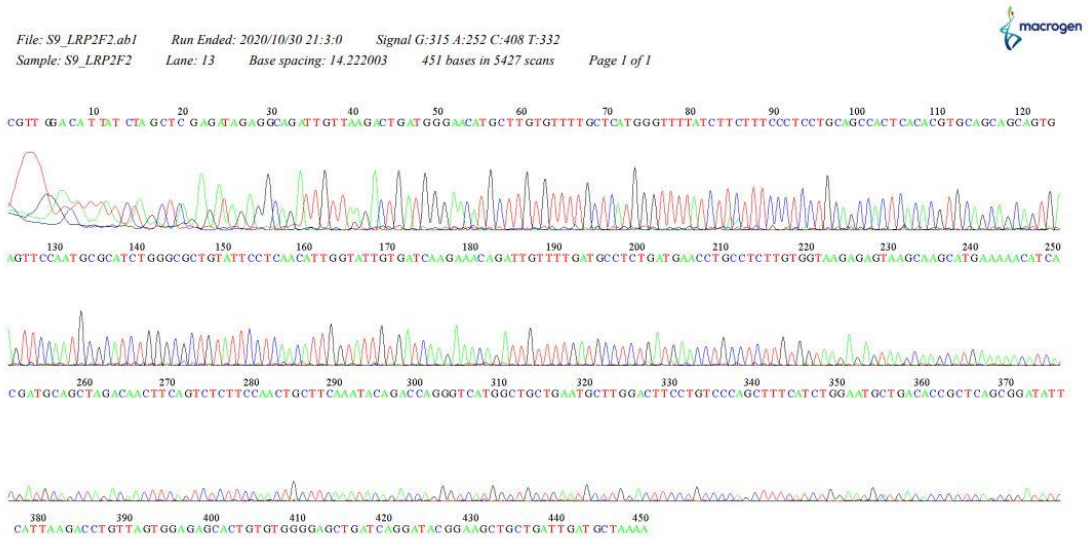


Figure 38 Chromatogram of sequencing from PCR-LRP2 sample 7

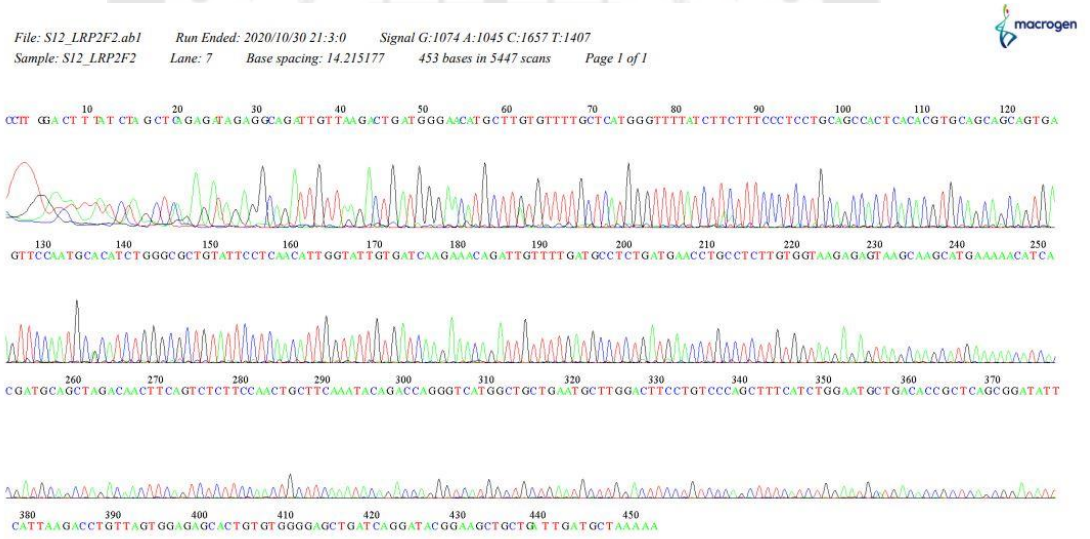


Figure 39 Chromatogram of sequencing from PCR-LRP2 sample 8

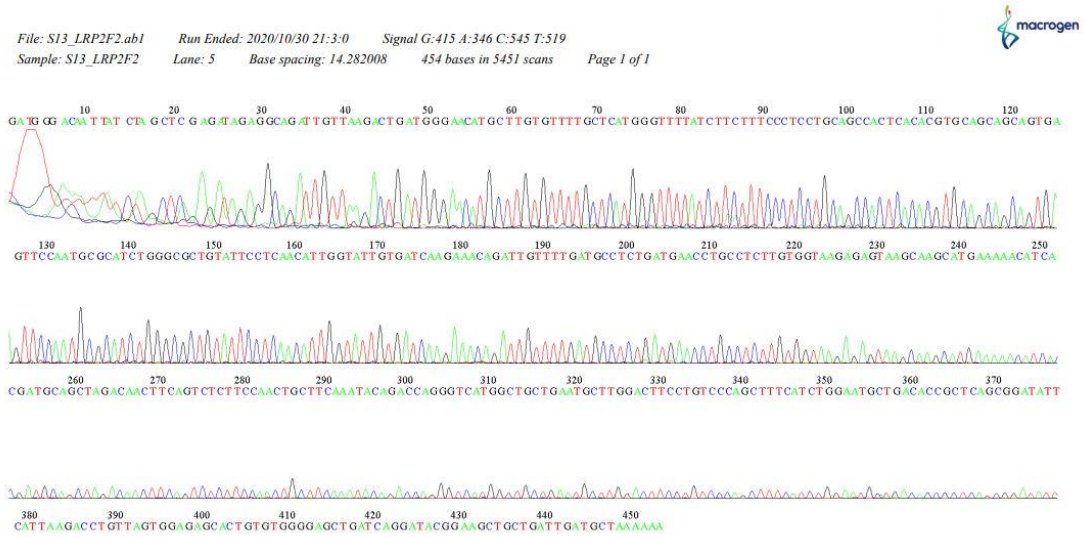


Figure 40 Chromatogram of sequencing from PCR-LRP2 sample 9

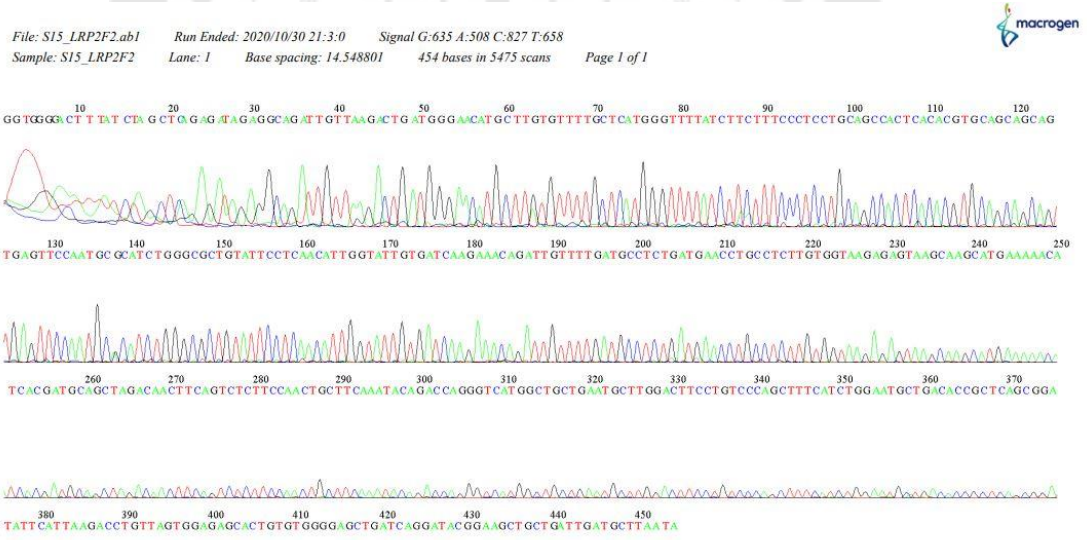


Figure 41 Chromatogram of sequencing from PCR-LRP2 sample 10

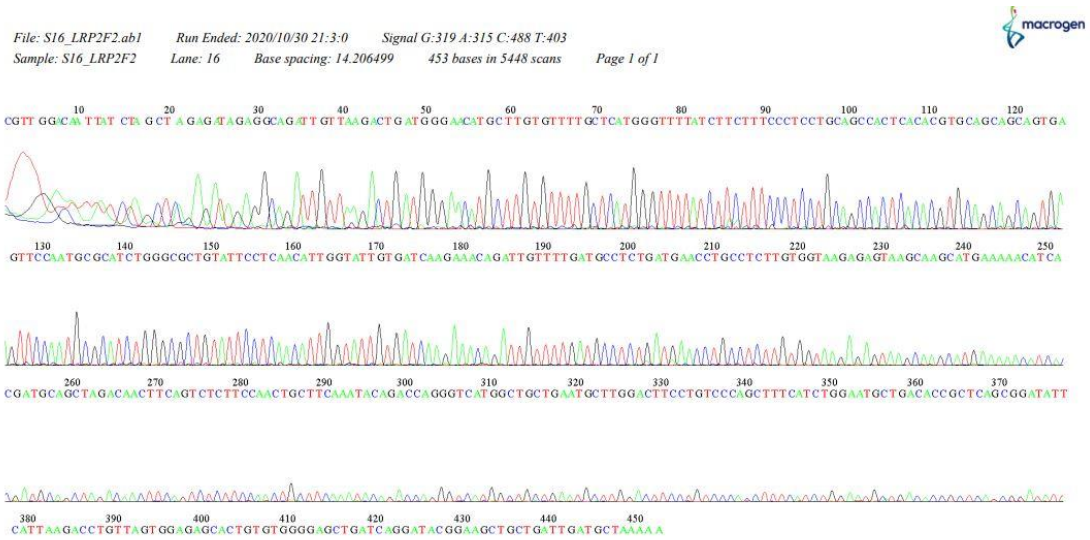


Figure 42 Chromatogram of sequencing from PCR-LRP2 sample 11

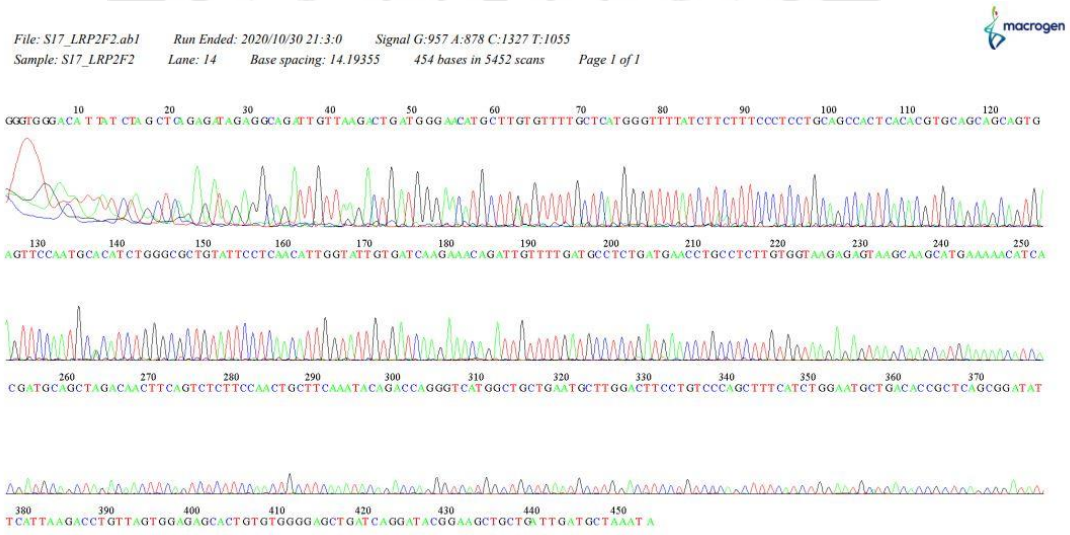


Figure 43 Chromatogram of sequencing from PCR-LRP2 sample 12

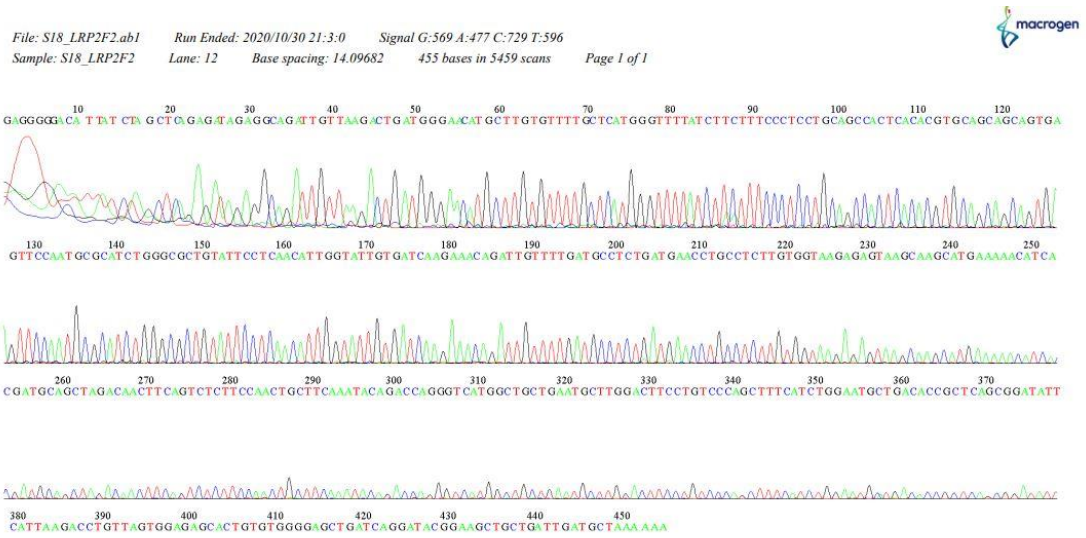


Figure 44 Chromatogram of sequencing from PCR-LRP2 sample 13

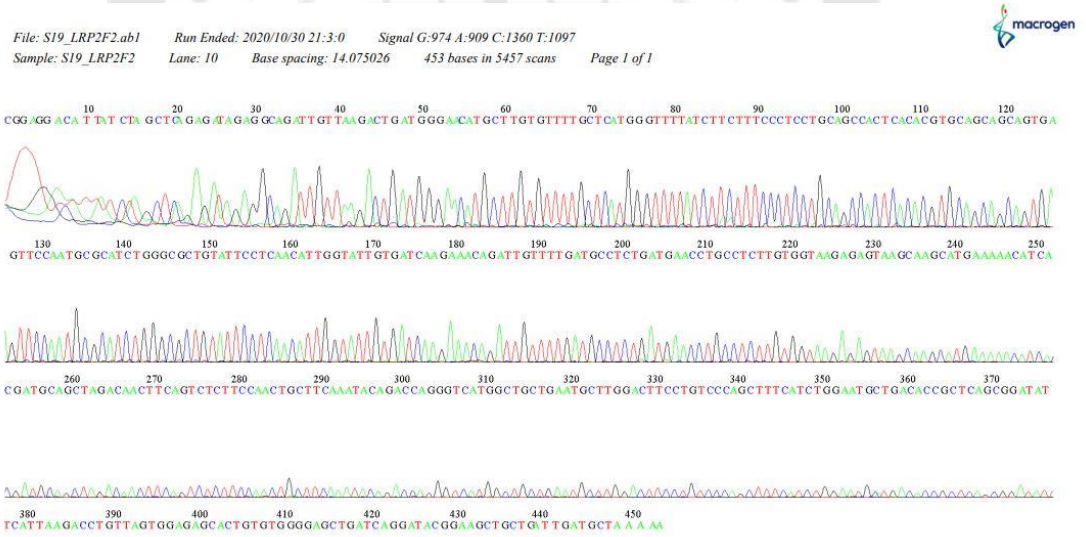


Figure 45 Chromatogram of sequencing from PCR-LRP2 sample 14

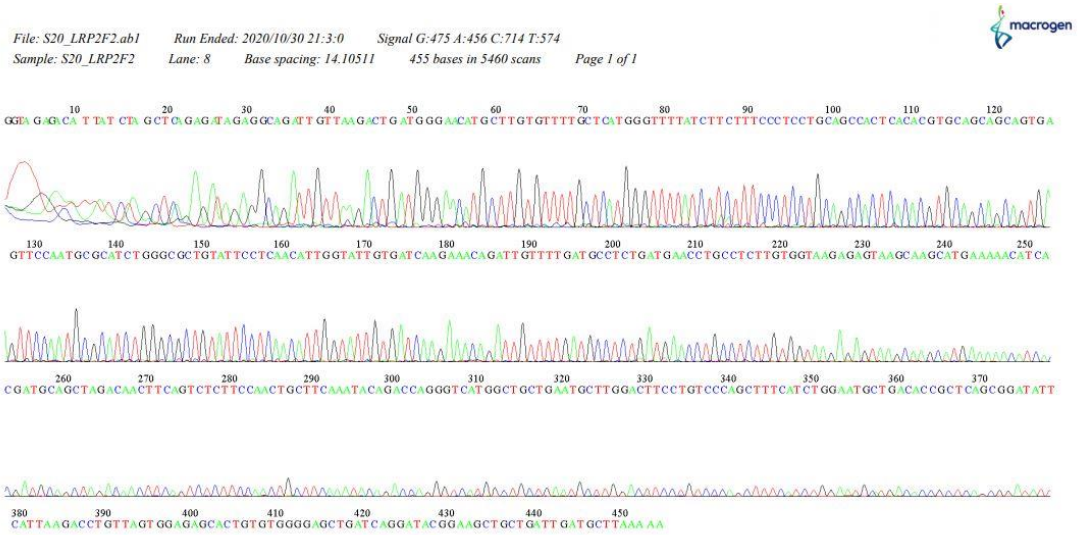


Figure 46 Chromatogram of sequencing from PCR-LRP2 sample 15

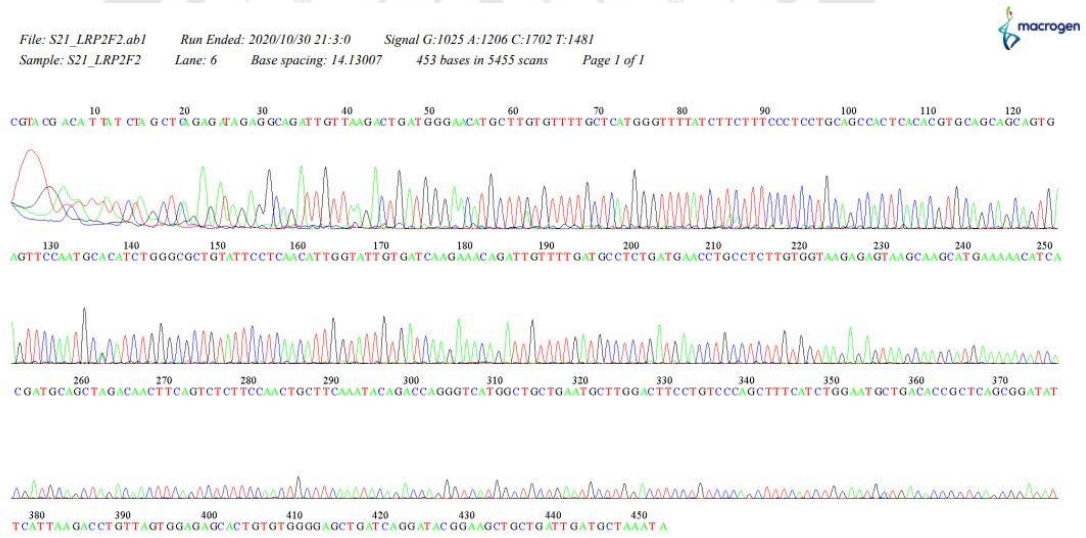


Figure 47 Chromatogram of sequencing from PCR-LRP2 sample 16

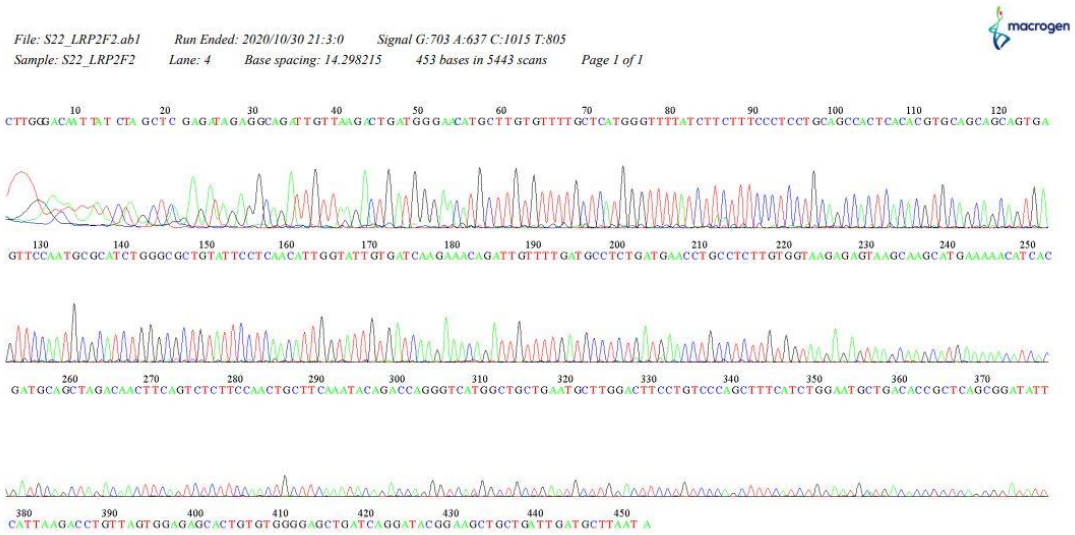


Figure 48 Chromatogram of sequencing from PCR-LRP2 sample 17

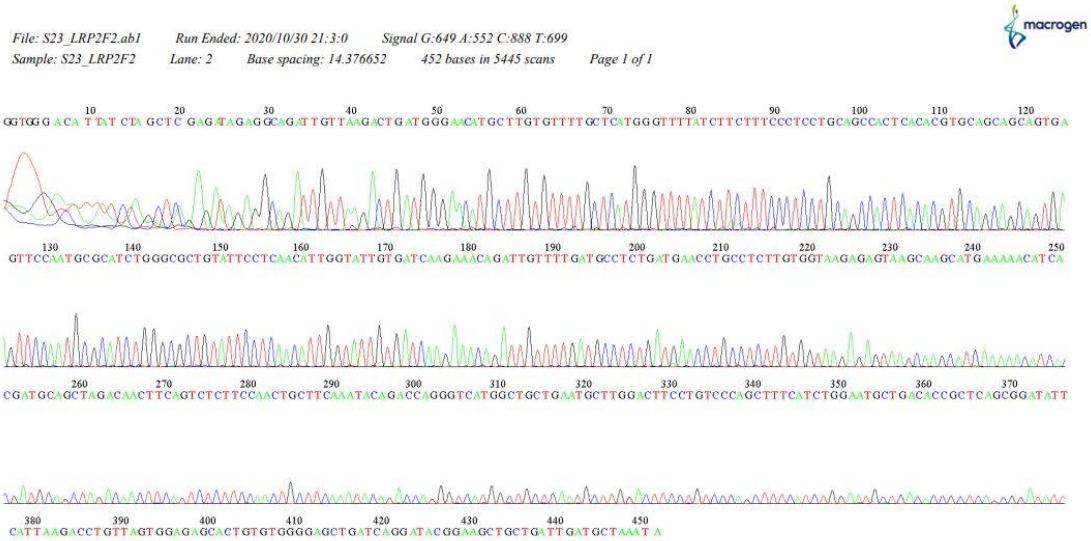


Figure 49 Chromatogram of sequencing from PCR-LRP2 sample 18

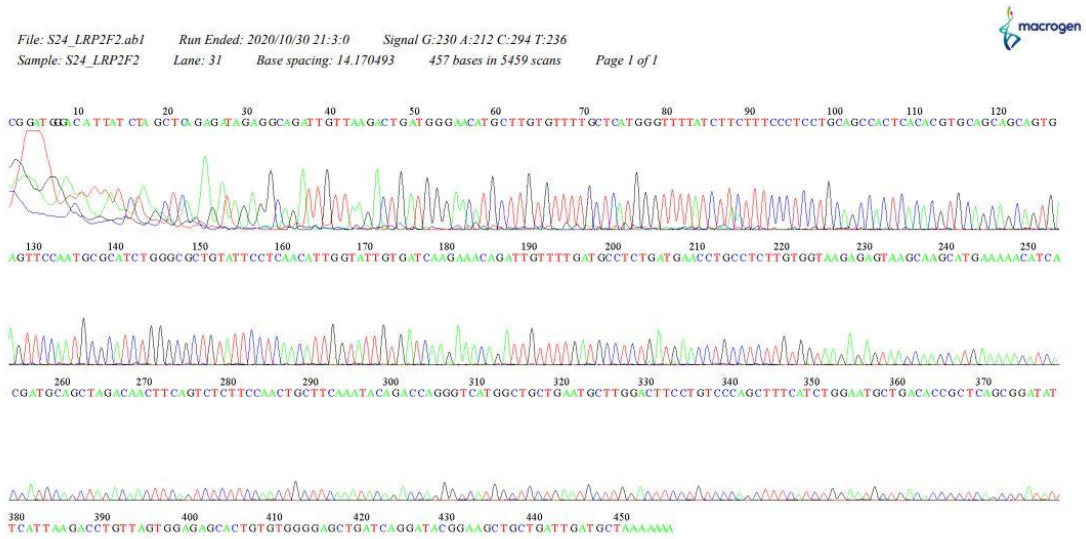


Figure 50 Chromatogram of sequencing from PCR-LRP2 sample 19

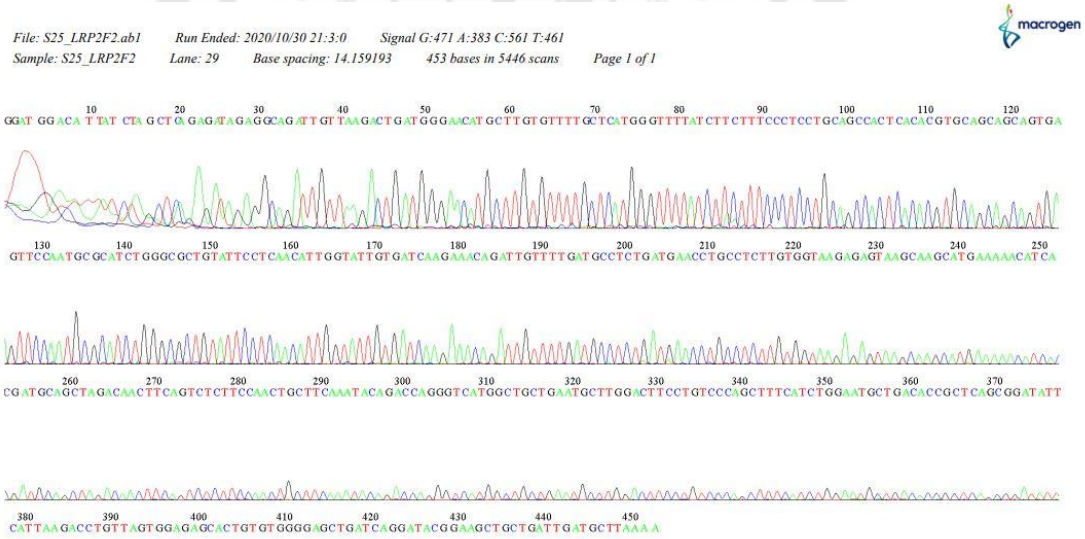


Figure 51 Chromatogram of sequencing from PCR-LRP2 sample 20

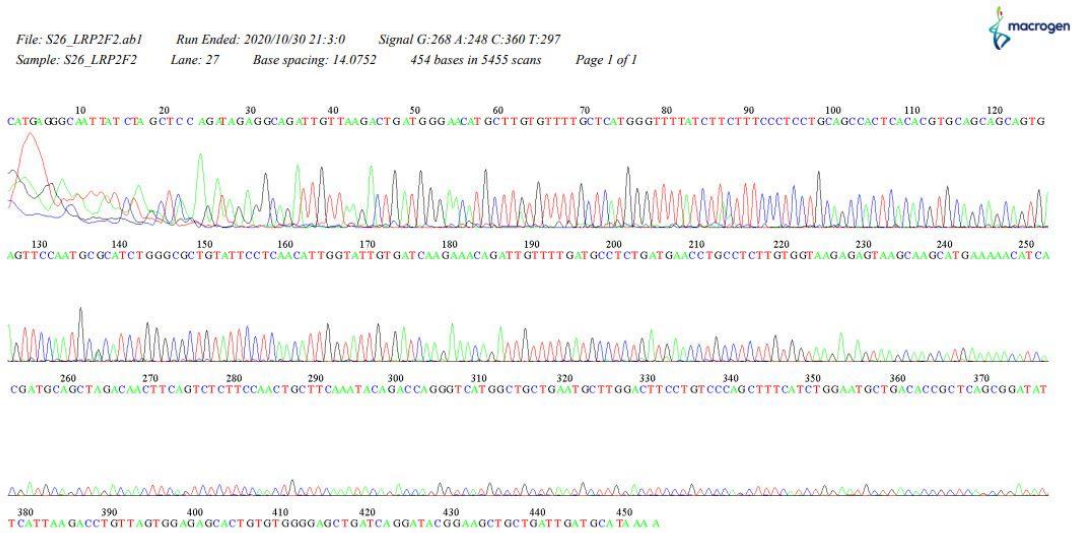


Figure 52 Chromatogram of sequencing from PCR-LRP2 sample 21

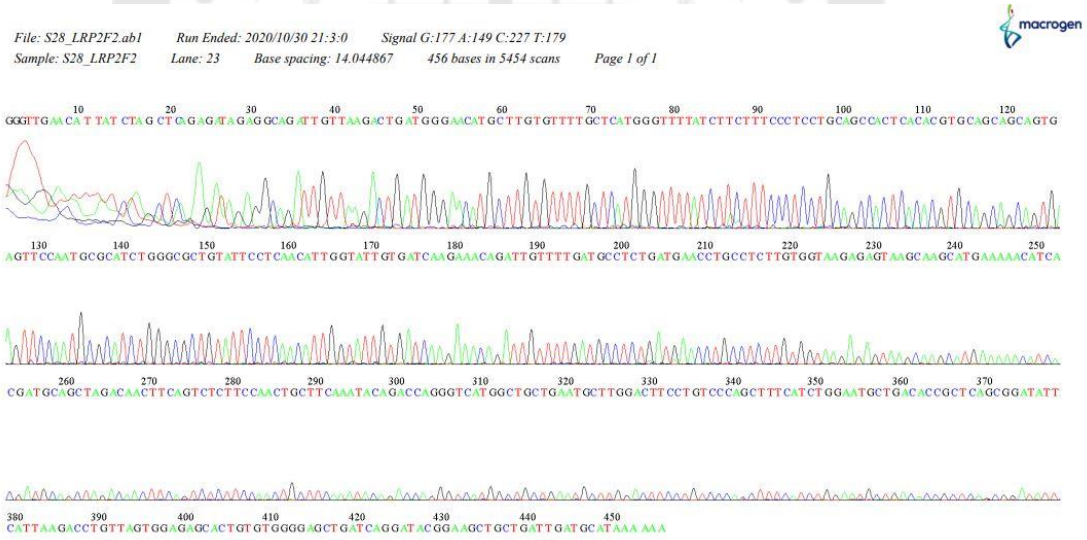


Figure 53 Chromatogram of sequencing from PCR-LRP2 sample 22

Figure 54 Chromatogram of sequencing from PCR-LRP2 sample 23

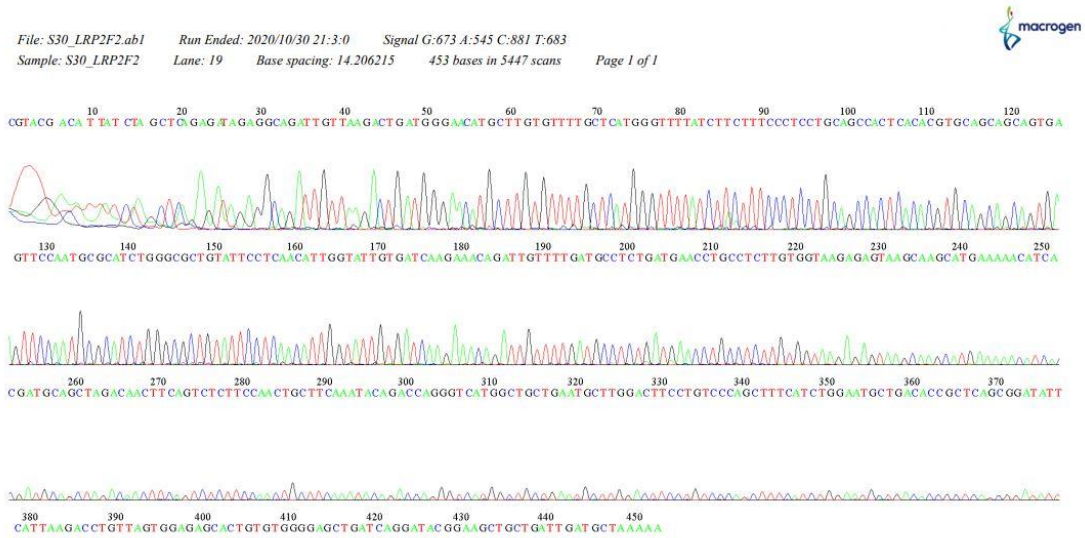


Figure 55 Chromatogram of sequencing from PCR-LRP2 sample 24

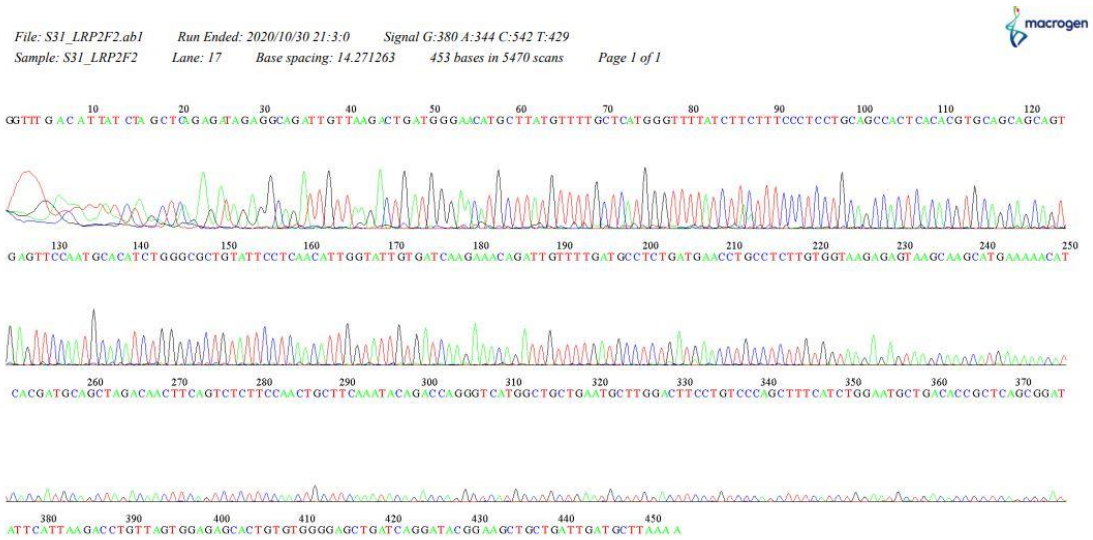


Figure 56 Chromatogram of sequencing from PCR-LRP2 sample 25

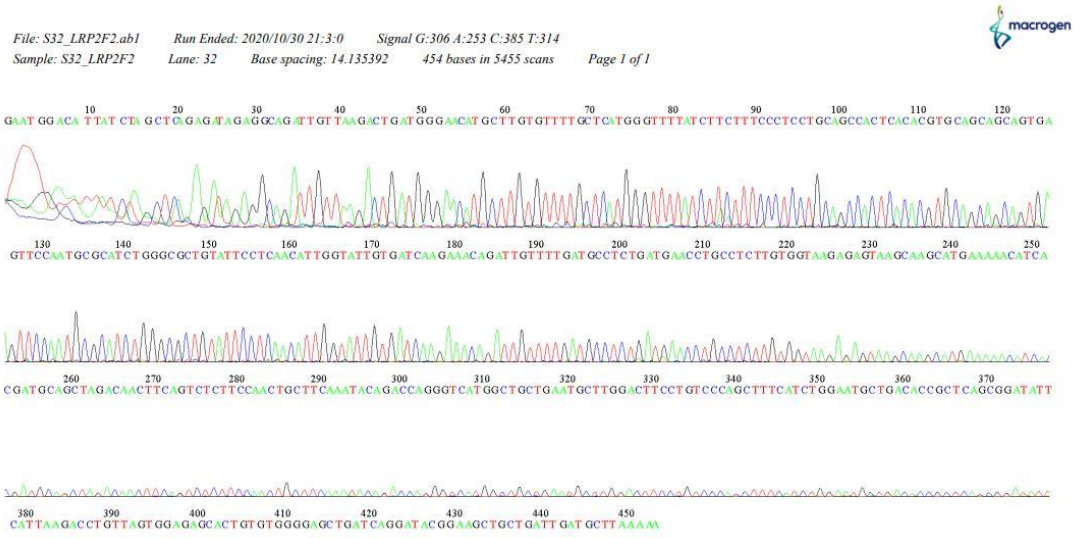


Figure 57 Chromatogram of sequencing from PCR-LRP2 sample 26

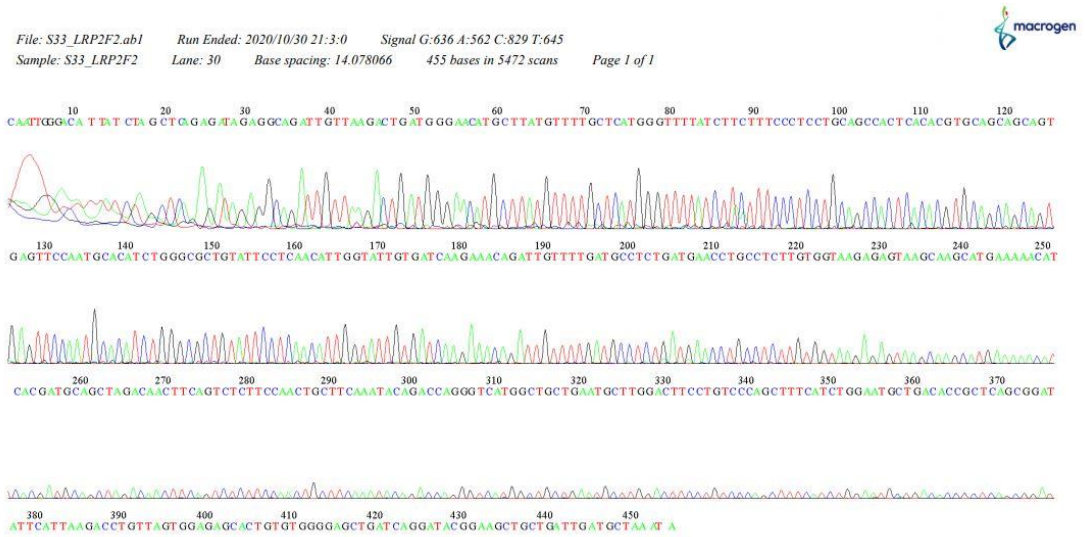


Figure 58 Chromatogram of sequencing from PCR-LRP2 sample 27

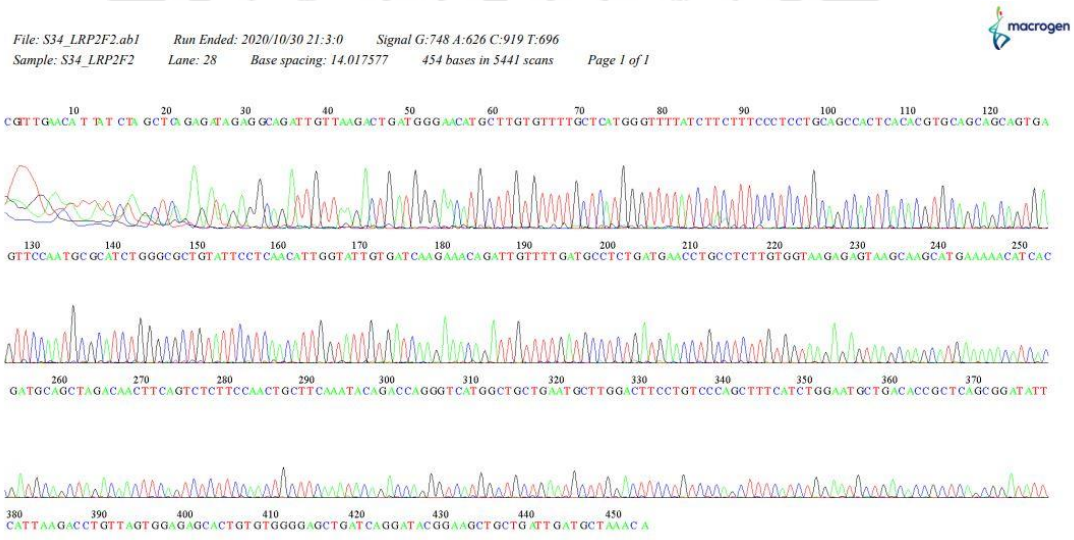


Figure 59 Chromatogram of sequencing from PCR-LRP2 sample 28

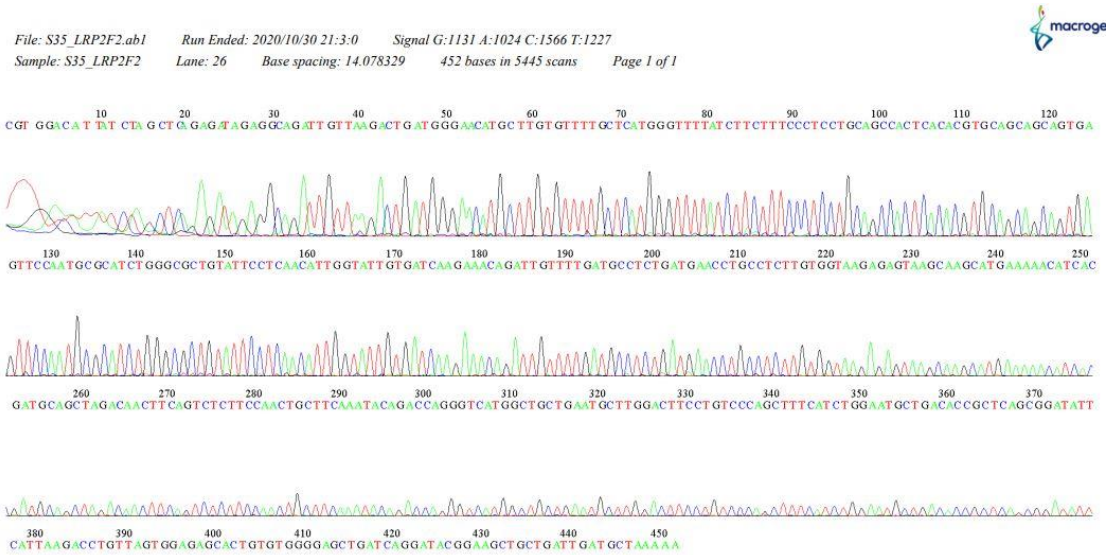


Figure 60 Chromatogram of sequencing from PCR-LRP2 sample 29

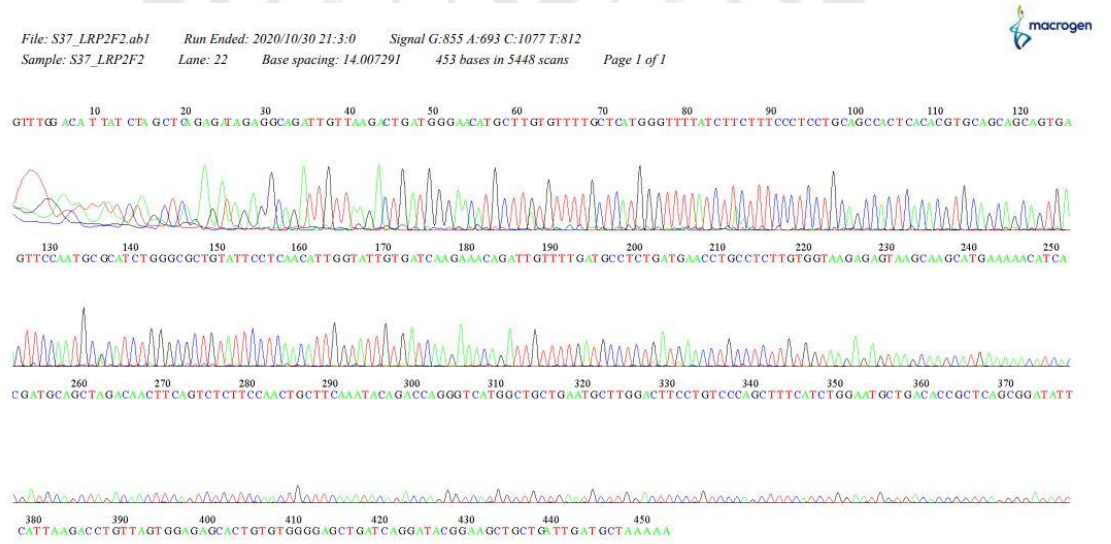


Figure 61 Chromatogram of sequencing from PCR-LRP2 sample 30

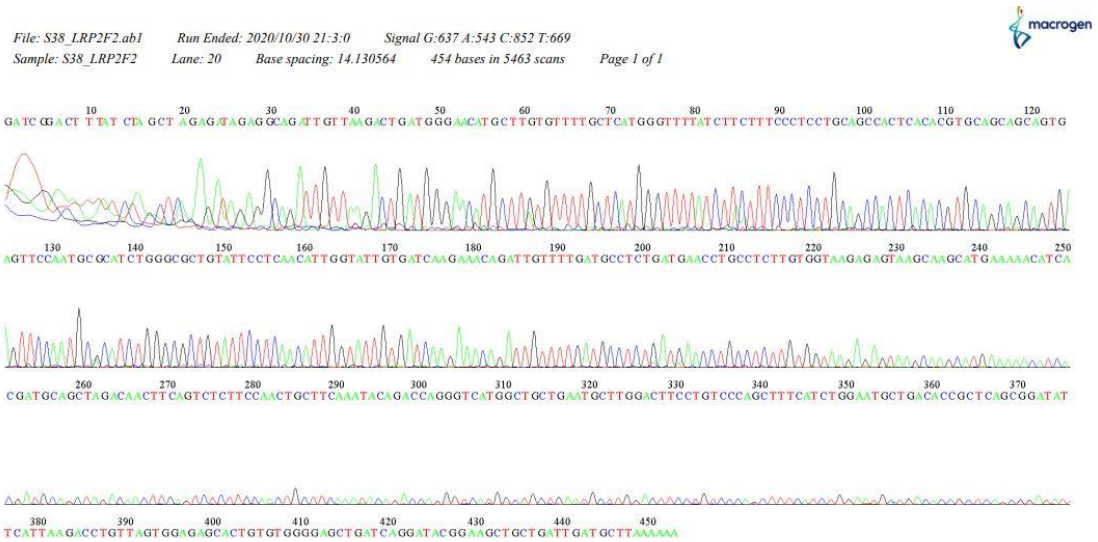


Figure 62 Chromatogram of sequencing from PCR-LRP2 sample 31

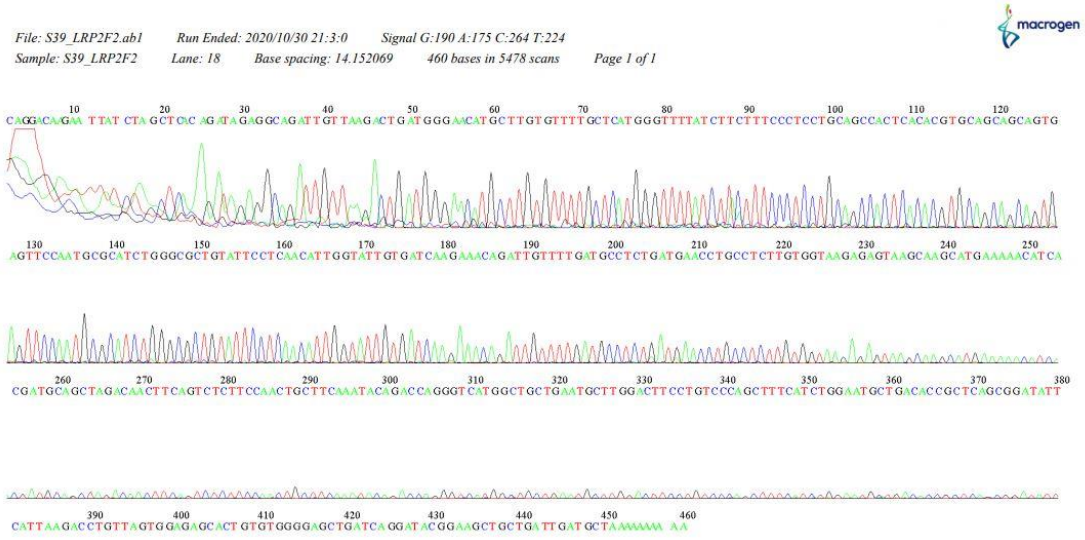


Figure 63 Chromatogram of sequencing from PCR-LRP2 sample 32

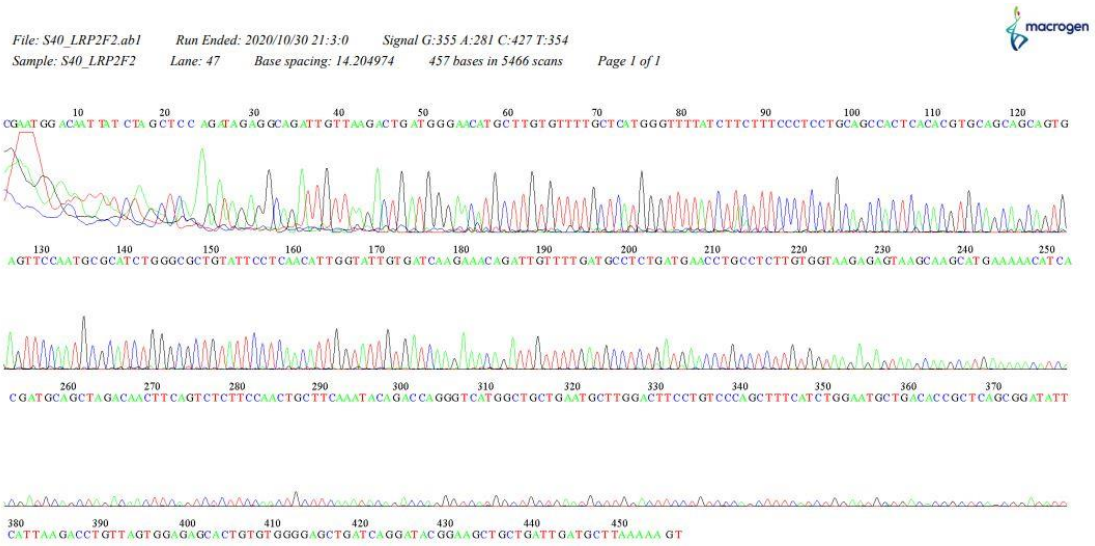


Figure 64 Chromatogram of sequencing from PCR-LRP2 sample 33

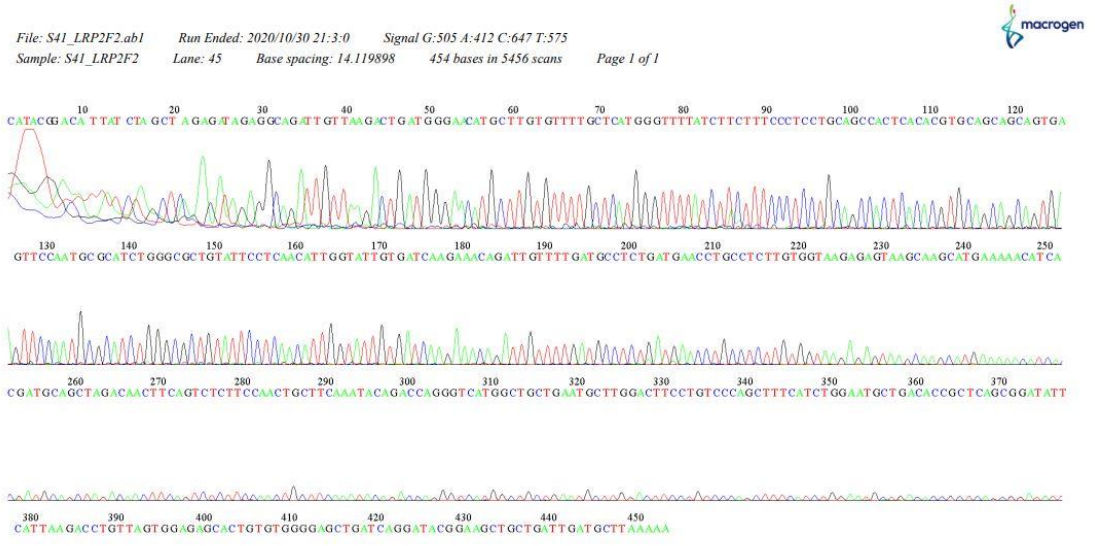


Figure 65 Chromatogram of sequencing from PCR-LRP2 sample 34

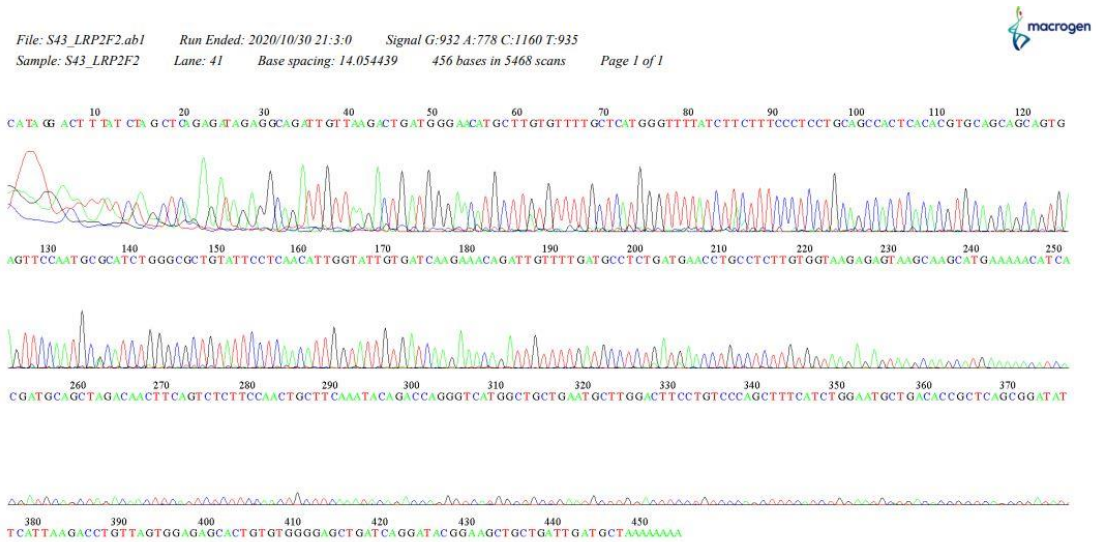


Figure 66 Chromatogram of sequencing from PCR-LRP2 sample 35

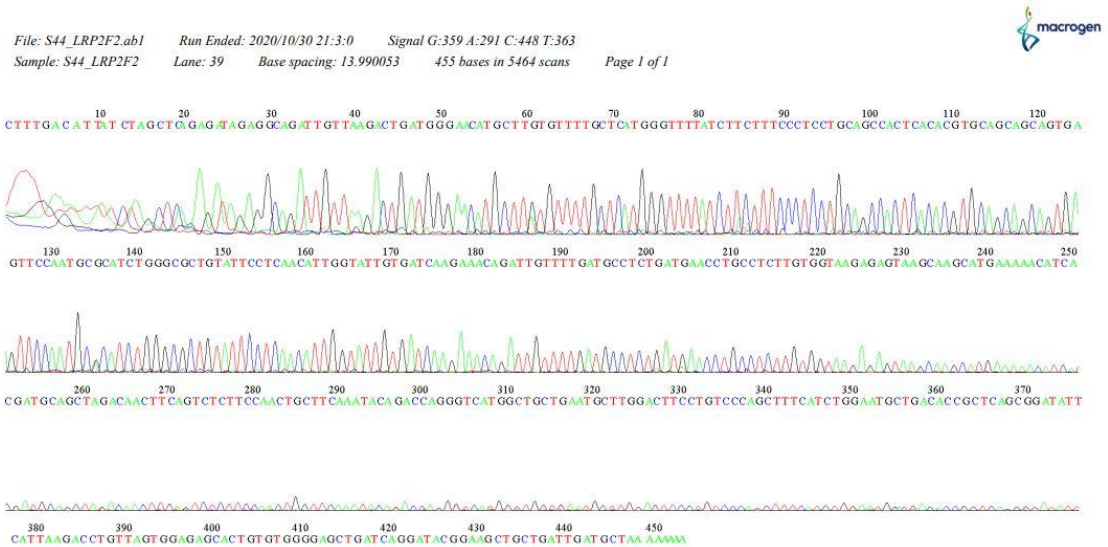


Figure 67 Chromatogram of sequencing from PCR-LRP2 sample 36

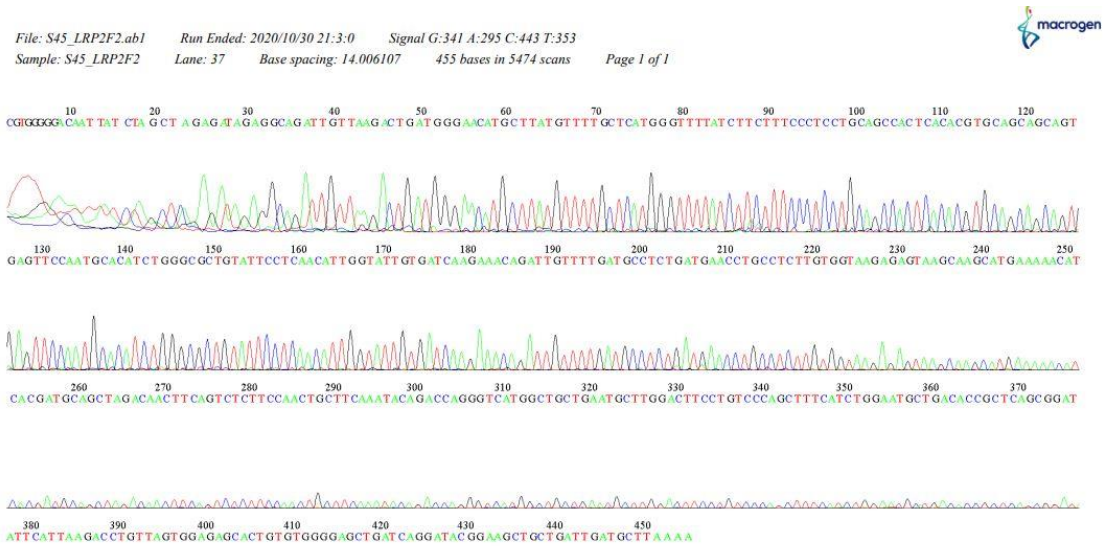


Figure 68 Chromatogram of sequencing from PCR-LRP2 sample 37

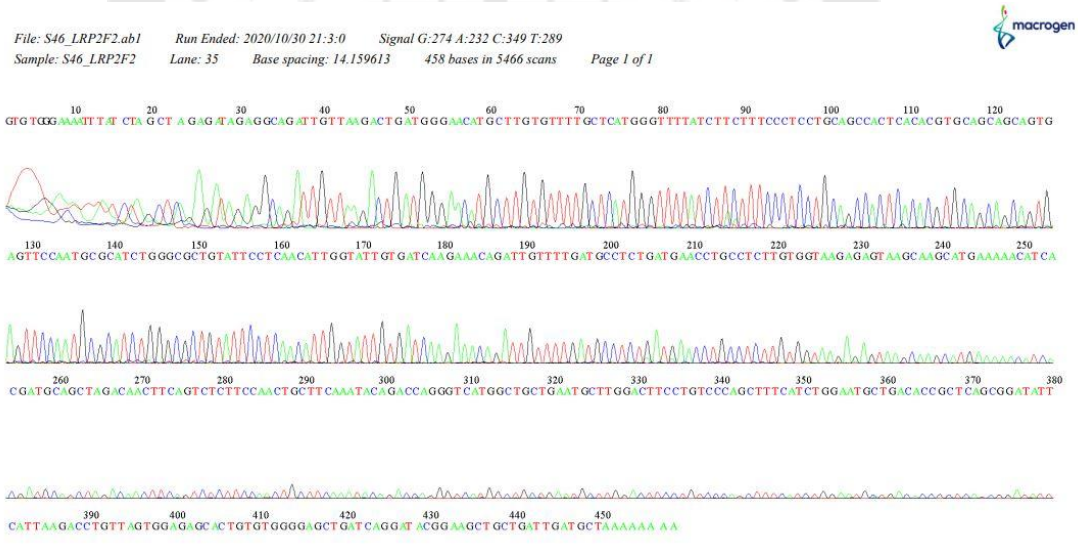


Figure 69 Chromatogram of sequencing from PCR-LRP2 sample 38

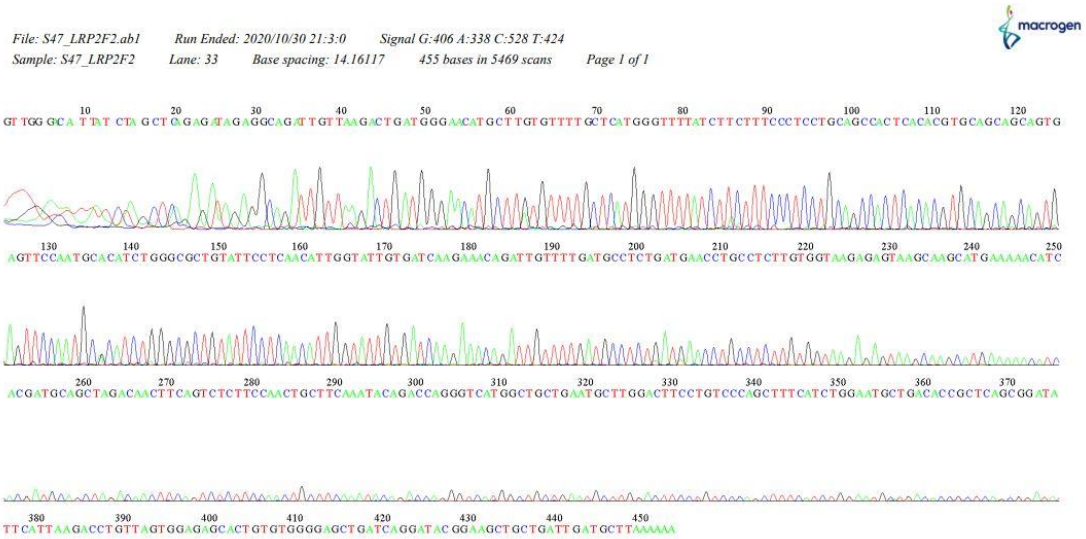


Figure 70 Chromatogram of sequencing from PCR-LRP2 sample 39

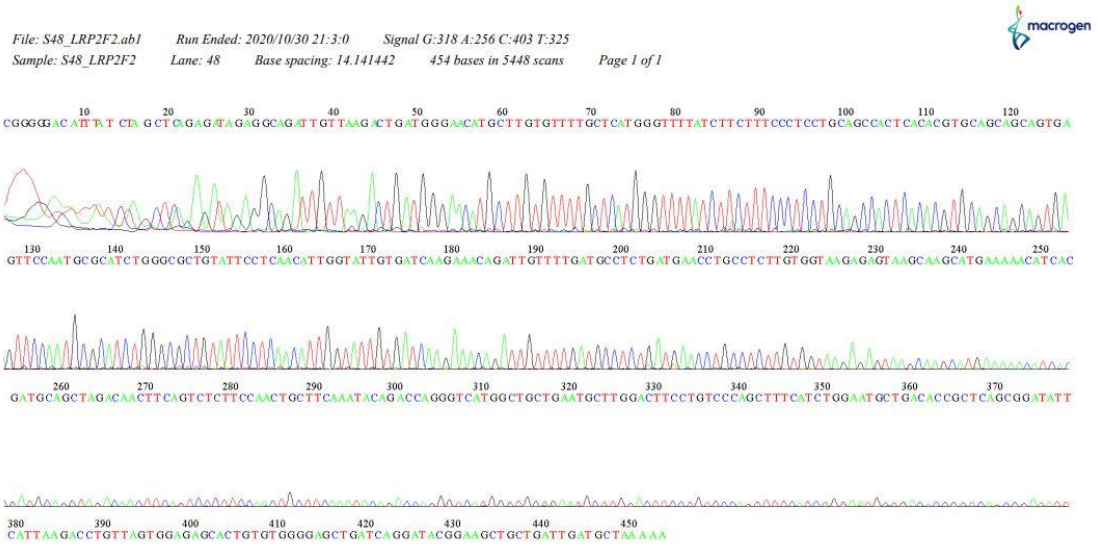


Figure 71 Chromatogram of sequencing from PCR-LRP2 sample 40

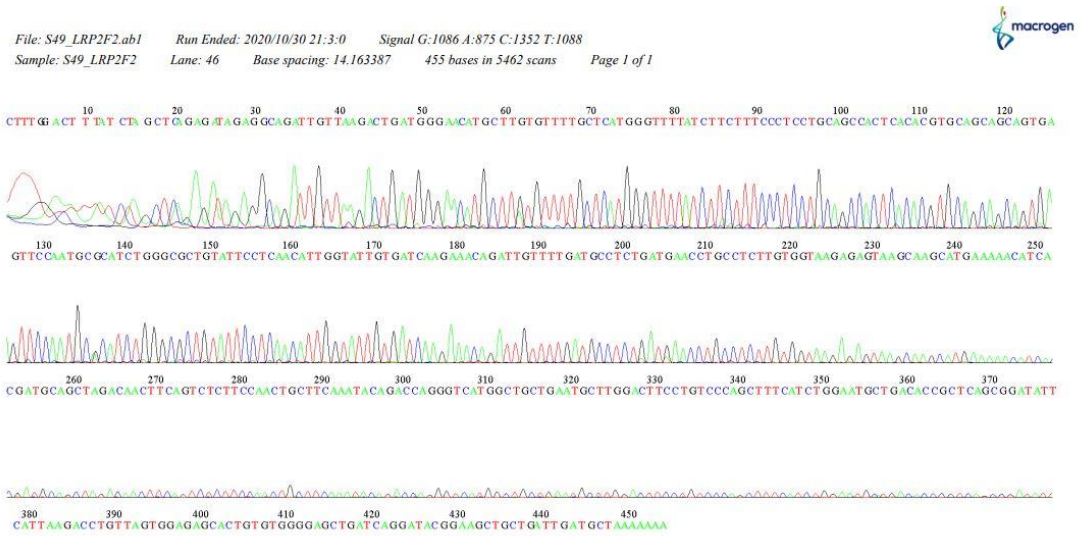


Figure 72 Chromatogram of sequencing from PCR-LRP2 sample 41

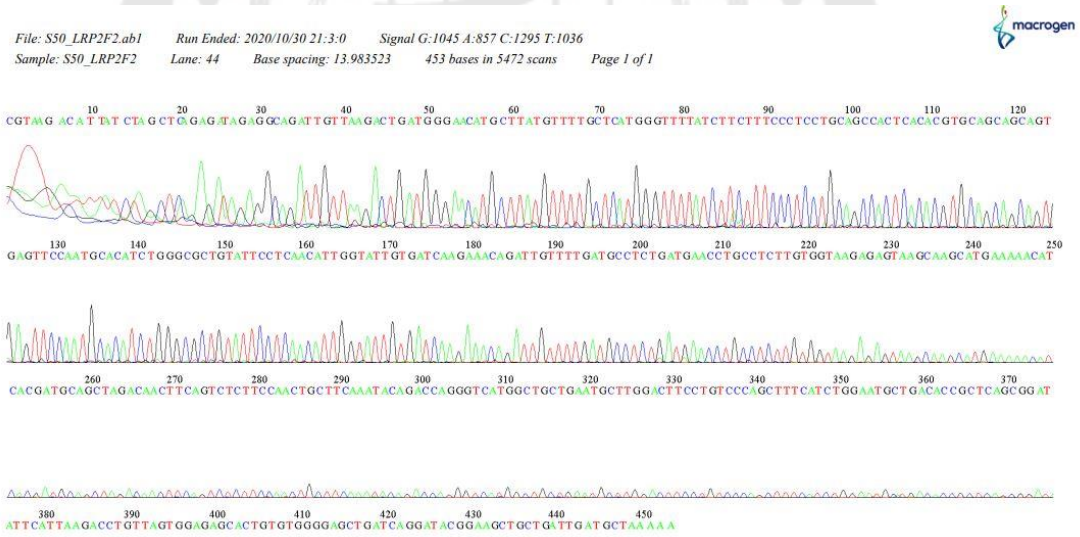


Figure 73 Chromatogram of sequencing from PCR-LRP2 sample 42

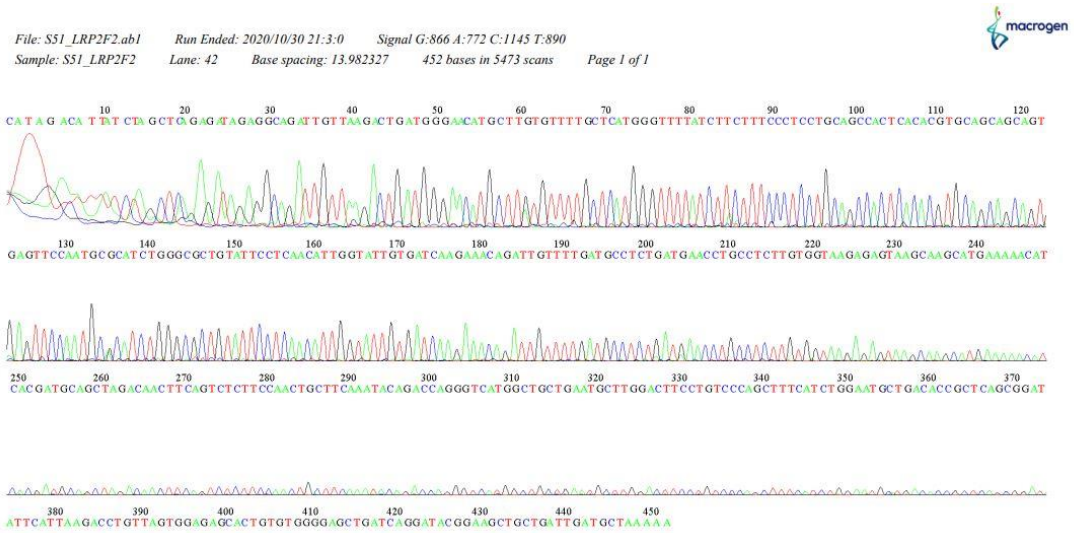


Figure 74 Chromatogram of sequencing from PCR-LRP2 sample 43

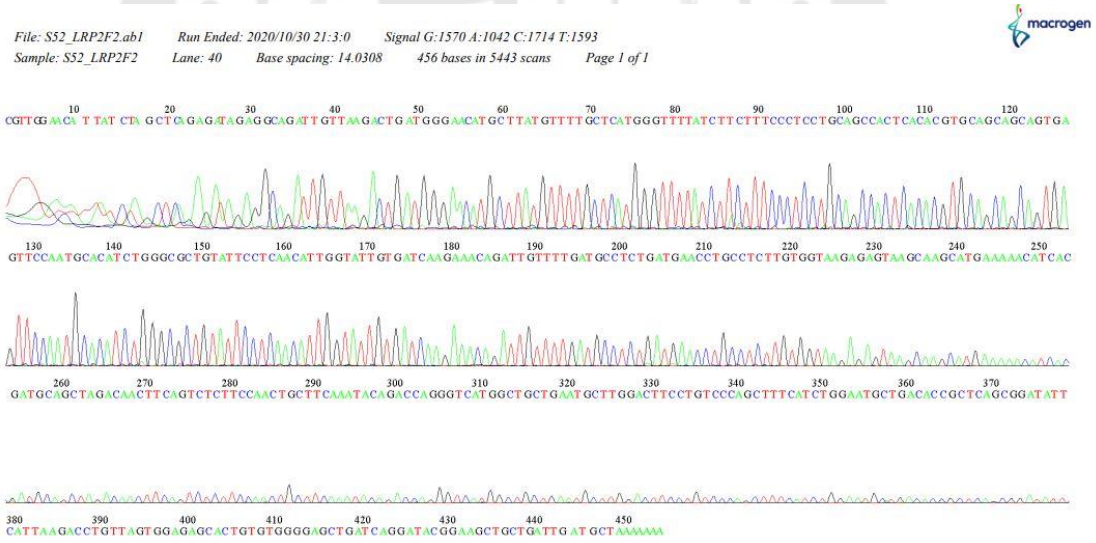


Figure 75 Chromatogram of sequencing from PCR-LRP2 sample 44

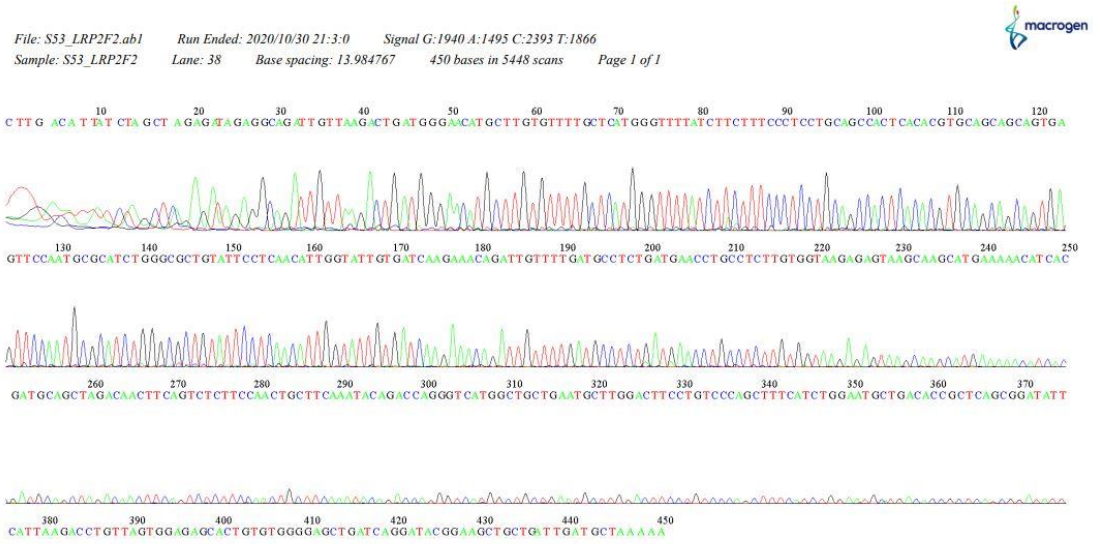


Figure 76 Chromatogram of sequencing from PCR-LRP2 sample 45

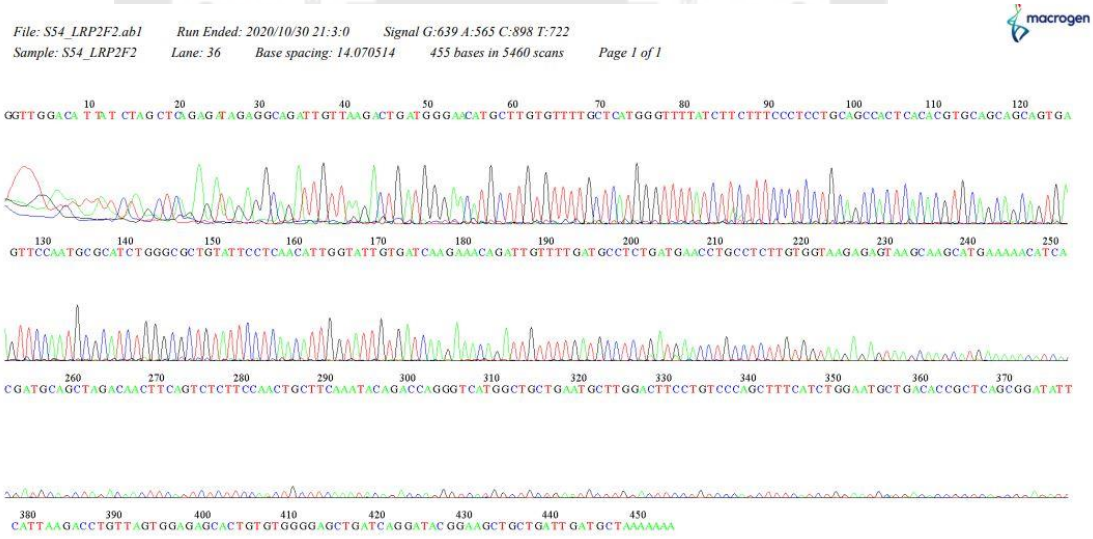


Figure 77 Chromatogram of sequencing from PCR-LRP2 sample 46

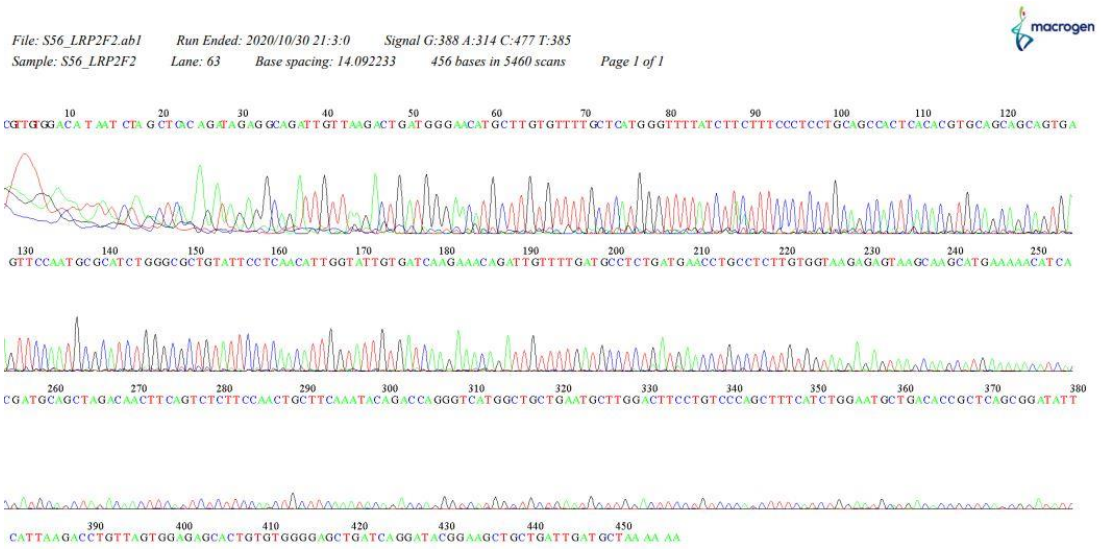


Figure 78 Chromatogram of sequencing from PCR-LRP2 sample 47

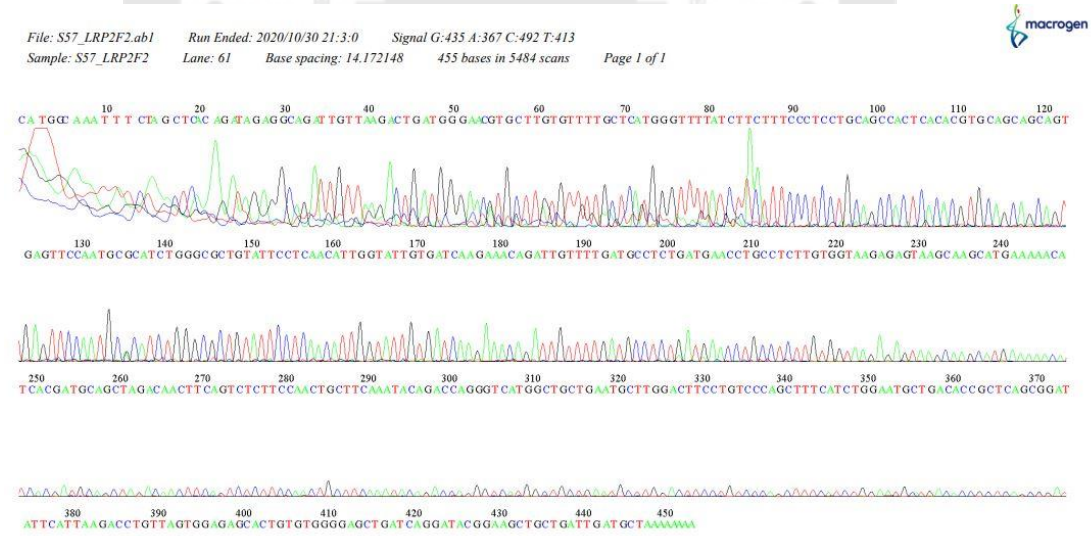


Figure 79 Chromatogram of sequencing from PCR-LRP2 sample 48

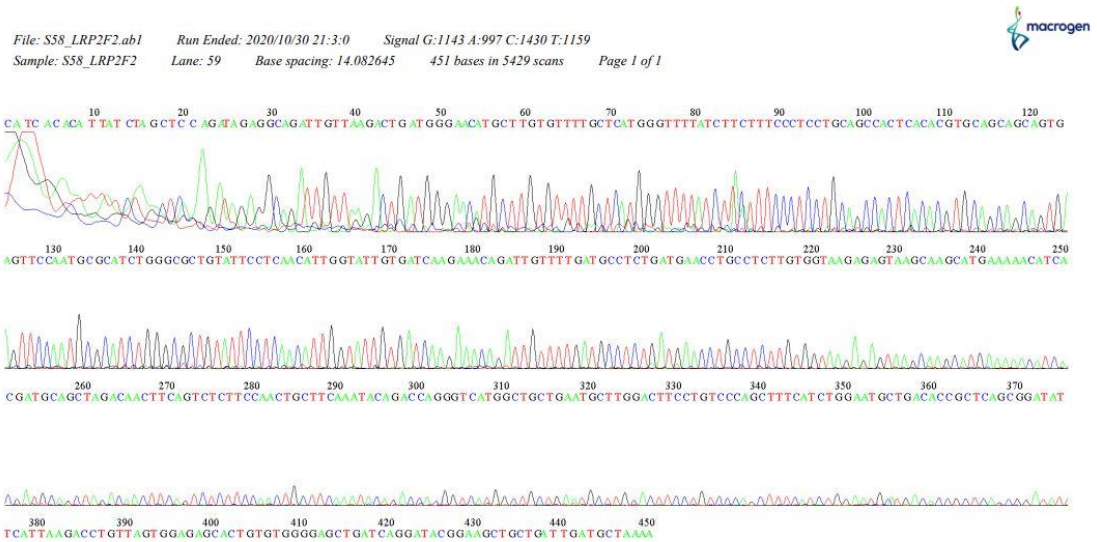


Figure 80 Chromatogram of sequencing from PCR-LRP2 sample 49

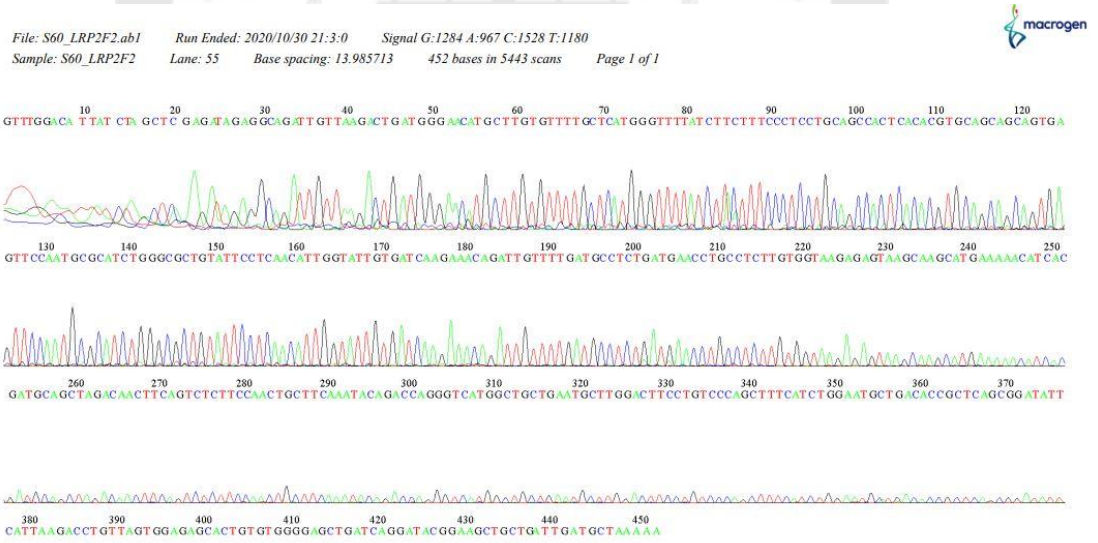


Figure 81 Chromatogram of sequencing from PCR-LRP2 sample 50

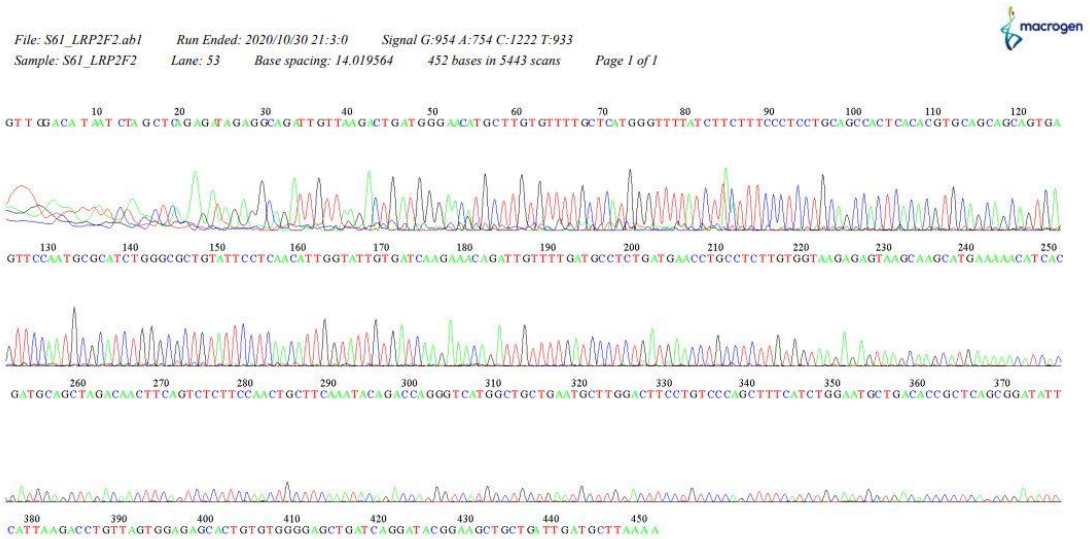


Figure 82 Chromatogram of sequencing from PCR-LRP2 sample 51

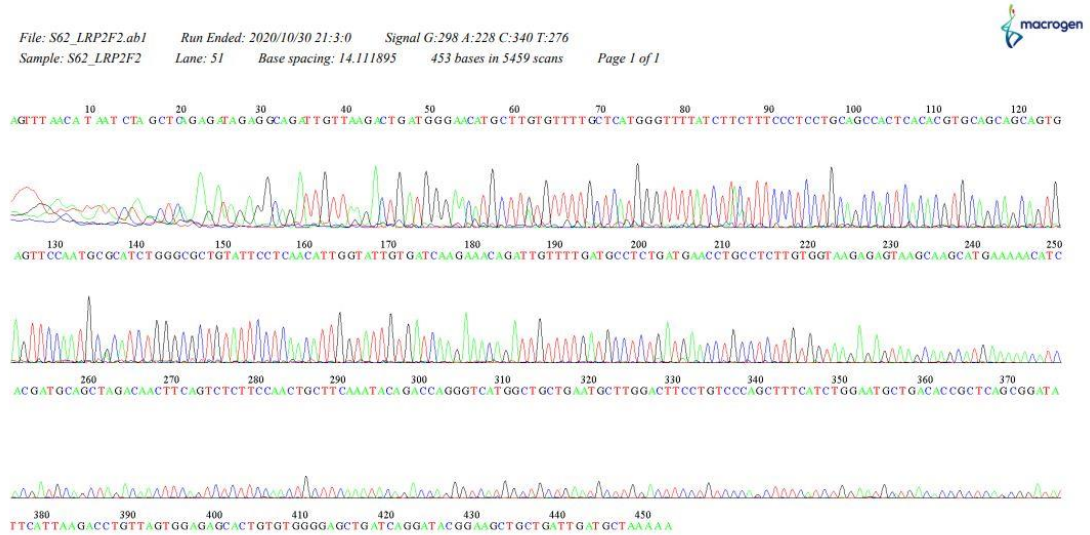


Figure 83 Chromatogram of sequencing from PCR-LRP2 sample 52

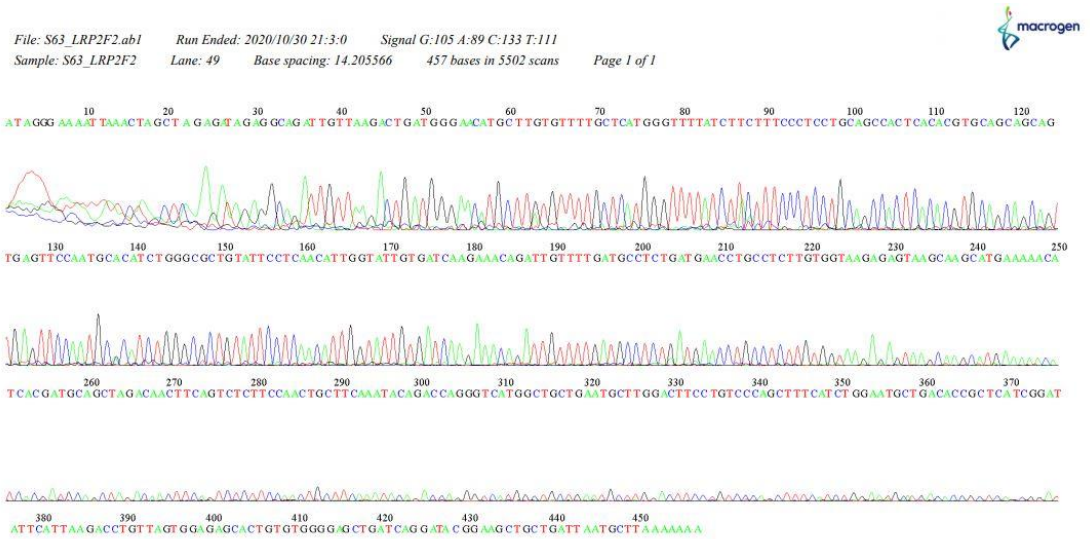


Figure 84 Chromatogram of sequencing from PCR-LRP2 sample 53

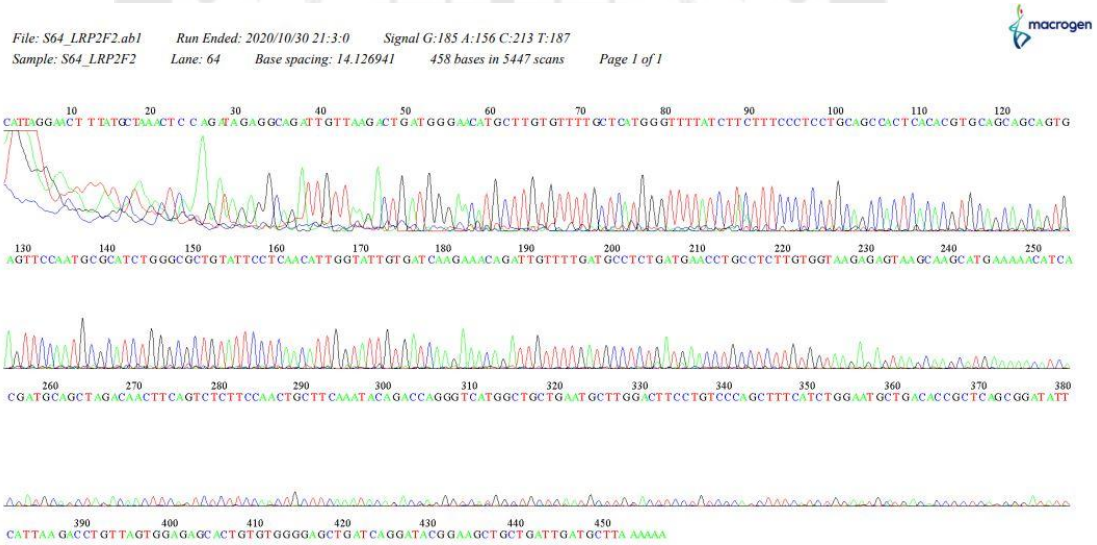


Figure 85 Chromatogram of sequencing from PCR-LRP2 sample 54

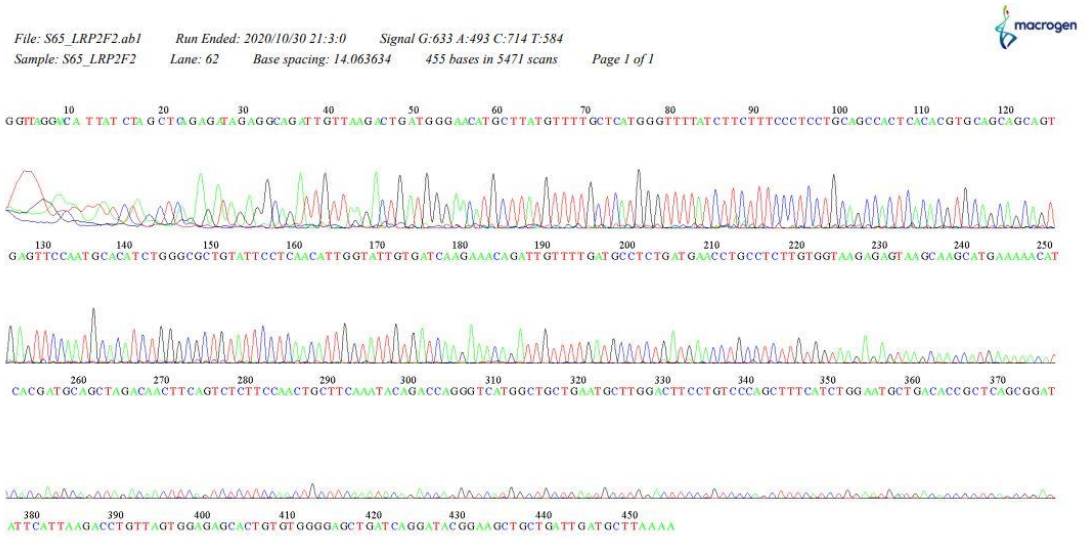


Figure 86 Chromatogram of sequencing from PCR-LRP2 sample 55

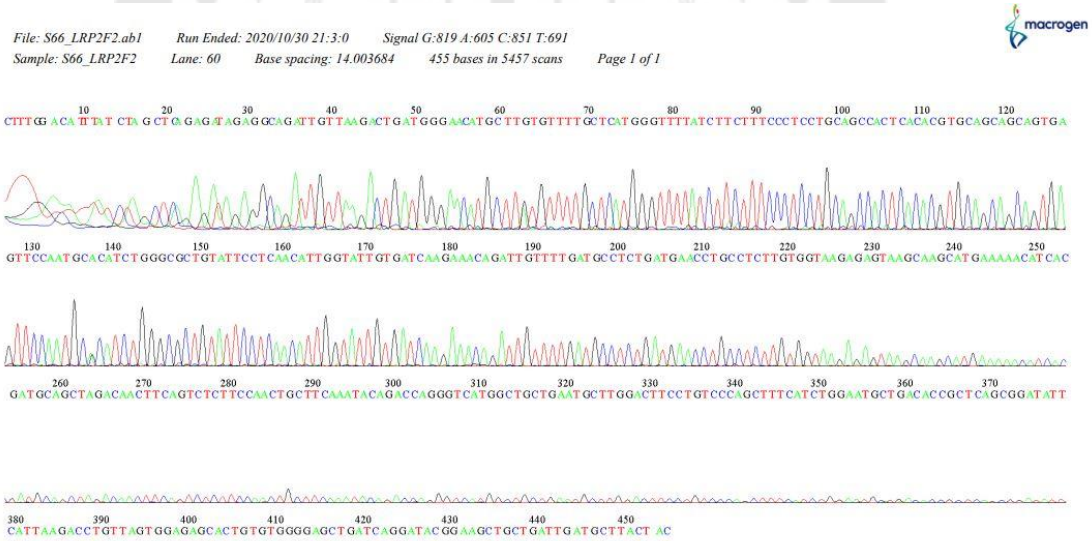


Figure 87 Chromatogram of sequencing from PCR-LRP2 sample 56

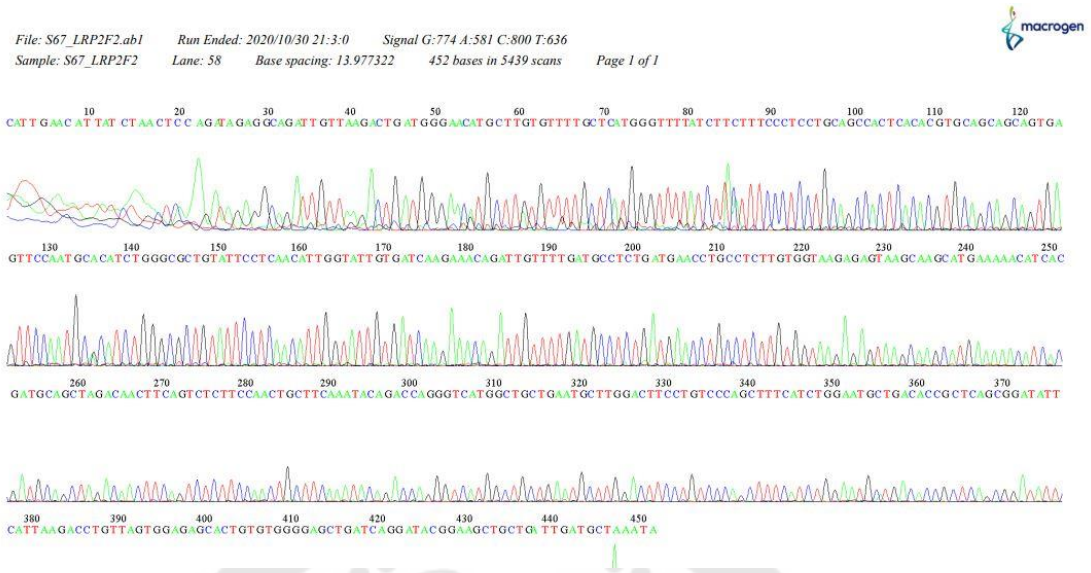


Figure 88 Chromatogram of sequencing from PCR-LRP2 sample 57

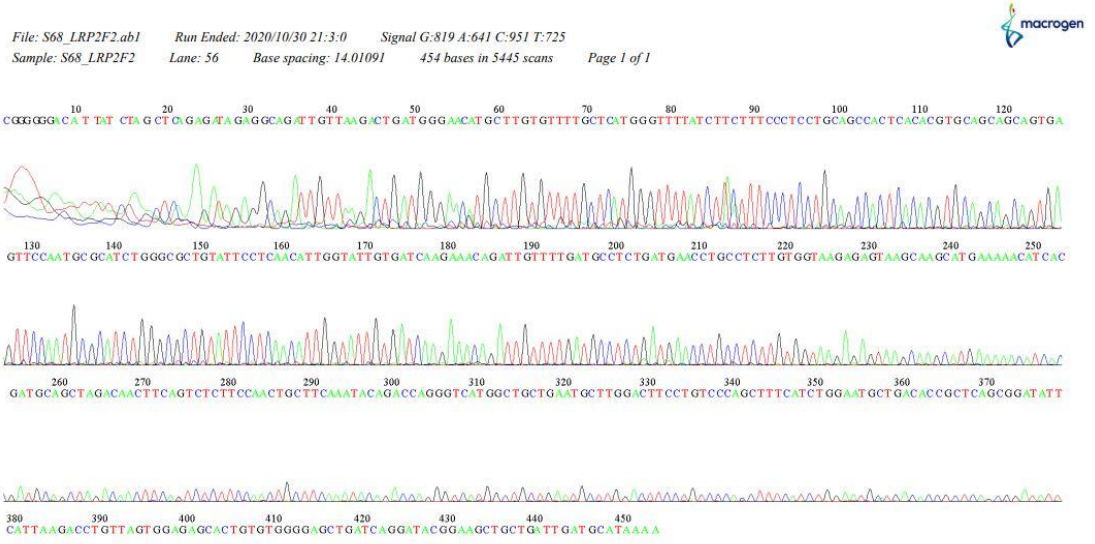


Figure 89 Chromatogram of sequencing from PCR-LRP2 sample 58

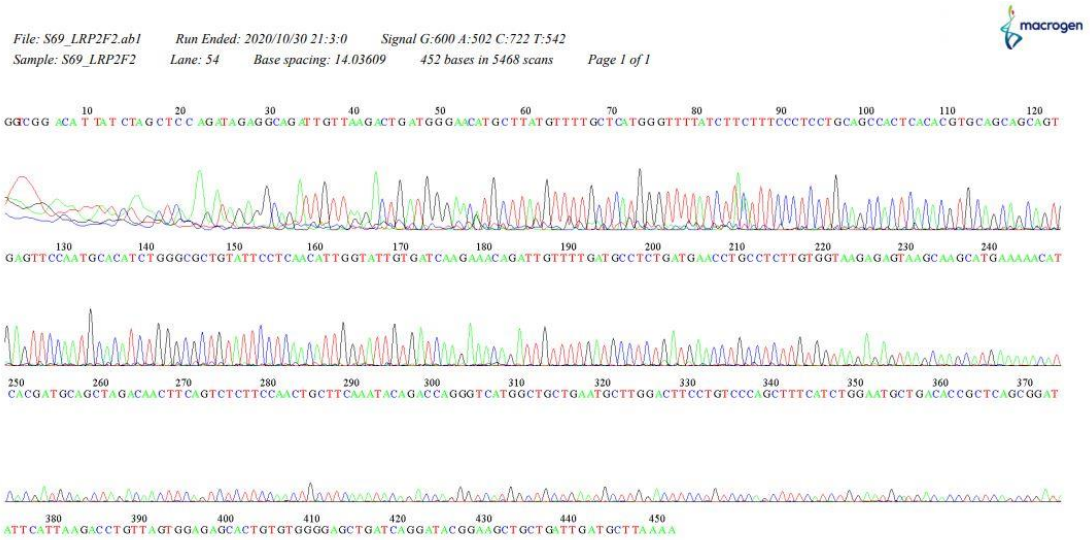


Figure 90 Chromatogram of sequencing from PCR-LRP2 sample 59

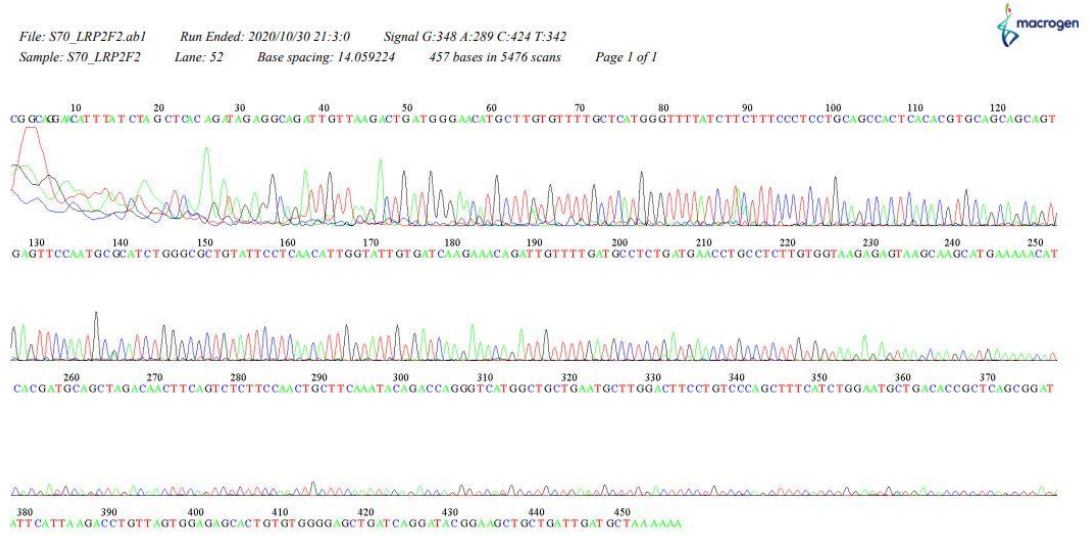


Figure 91 Chromatogram of sequencing from PCR-LRP2 sample 60

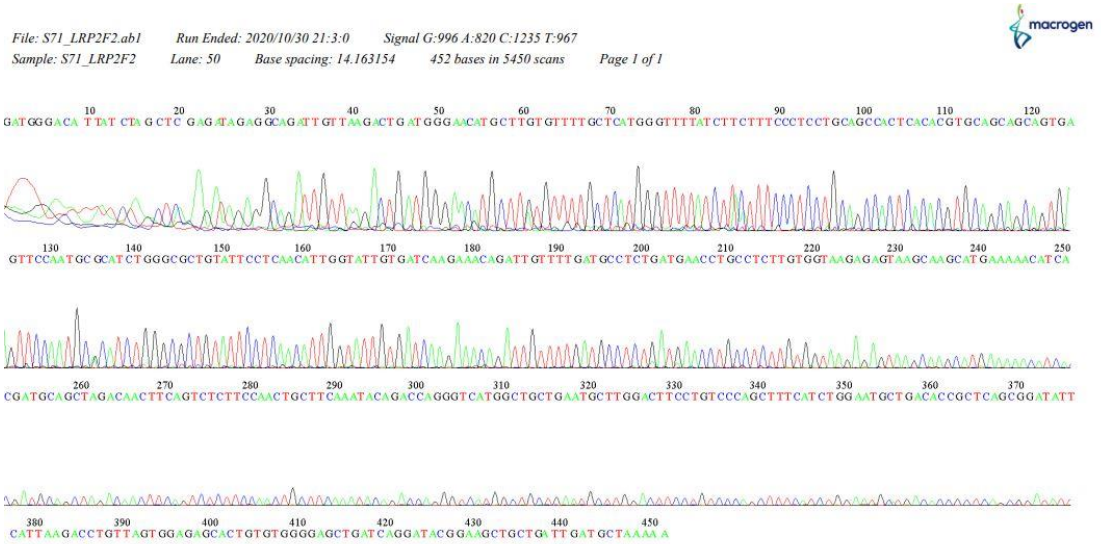


Figure 92 Chromatogram of sequencing from PCR-LRP2 sample 61

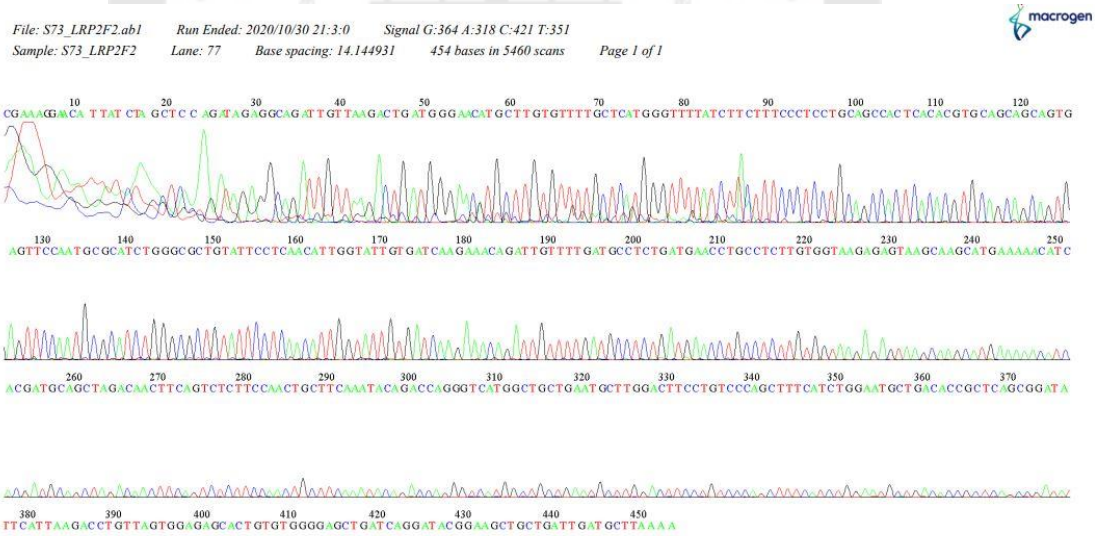


Figure 93 Chromatogram of sequencing from PCR-LRP2 sample 62

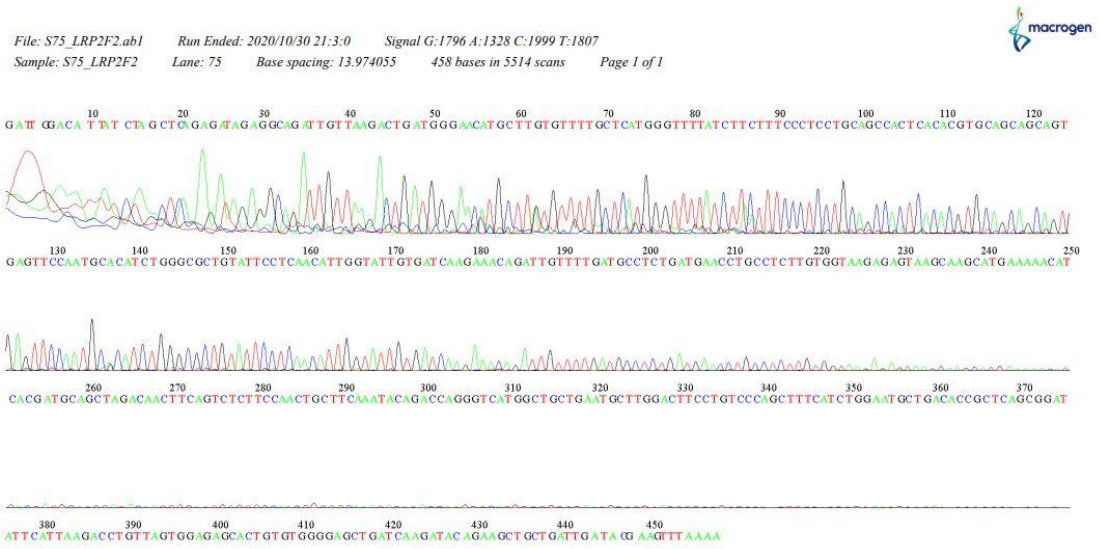


Figure 94 Chromatogram of sequencing from PCR-LRP2 sample 63

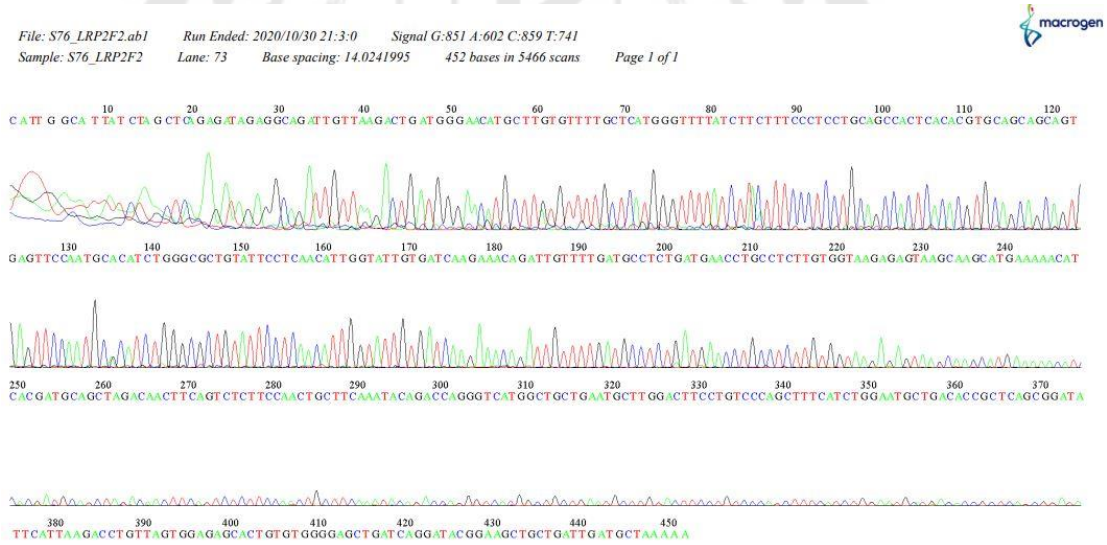


Figure 95 Chromatogram of sequencing from PCR-LRP2 sample 64

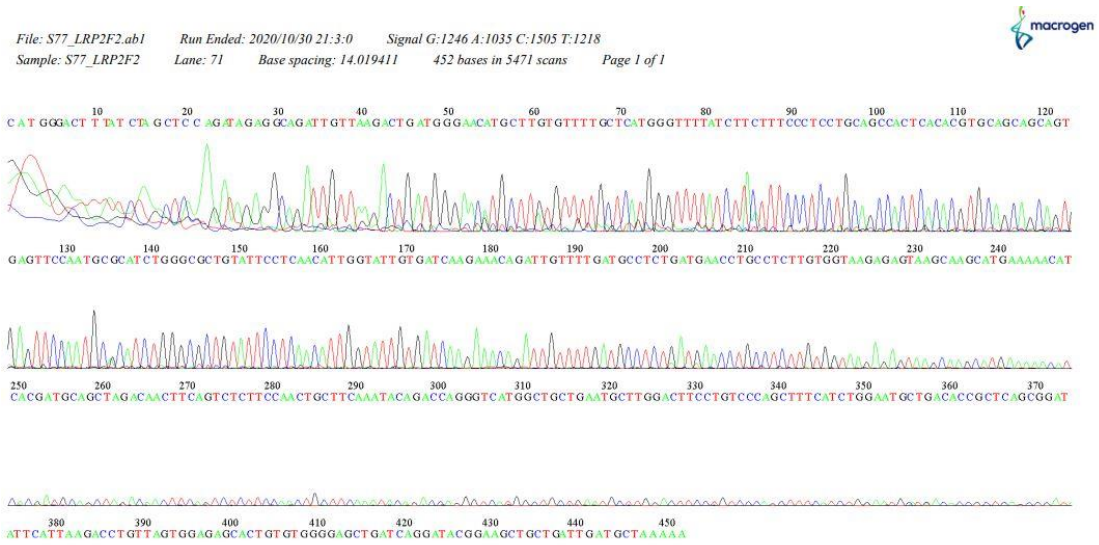


Figure 96 Chromatogram of sequencing from PCR-LRP2 sample 65

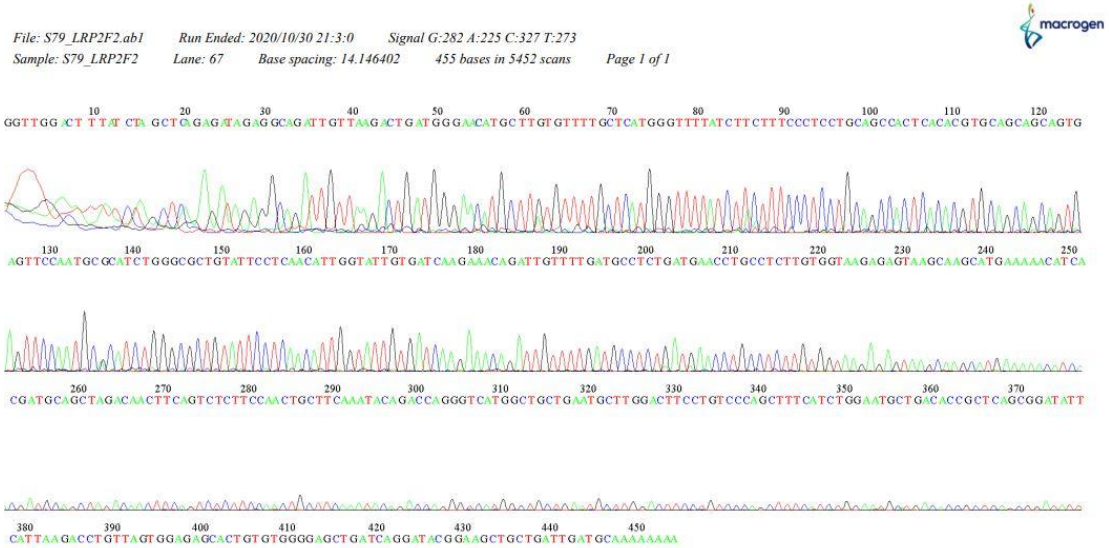


Figure 97 Chromatogram of sequencing from PCR-LRP2 sample 66

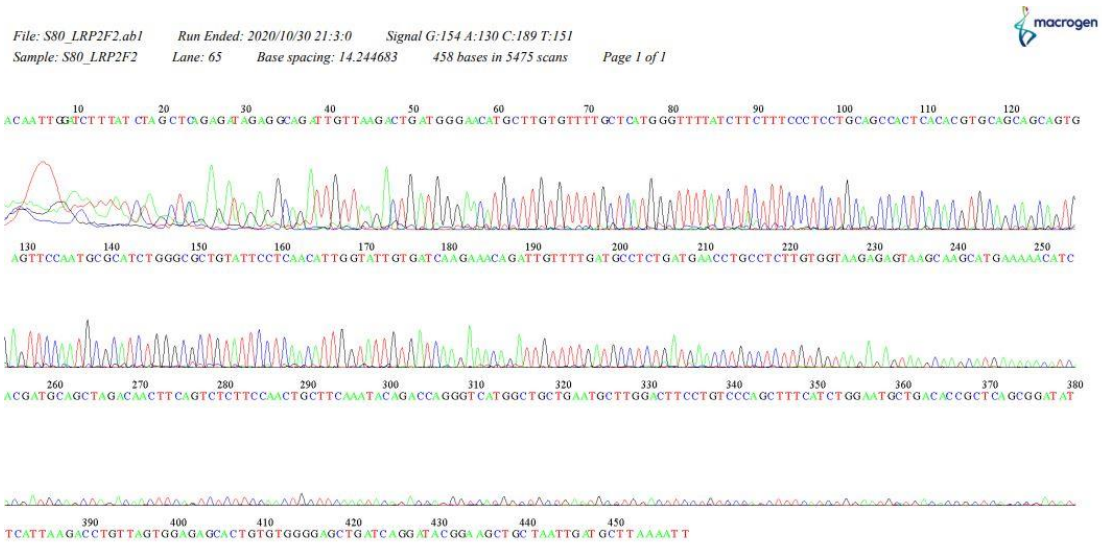


Figure 98 Chromatogram of sequencing from PCR-LRP2 sample 67

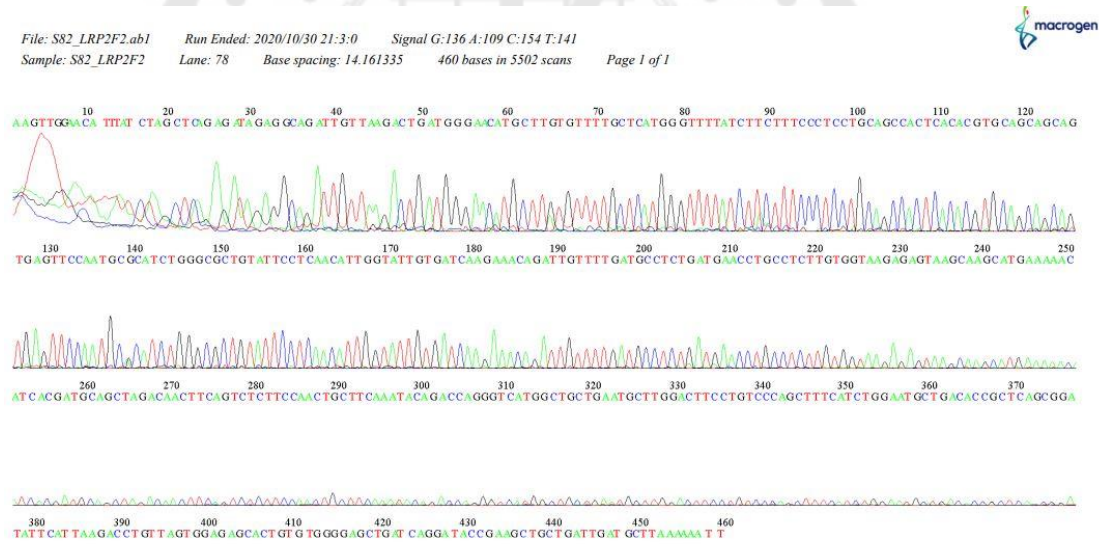


Figure 99 Chromatogram of sequencing from PCR-LRP2 sample 68

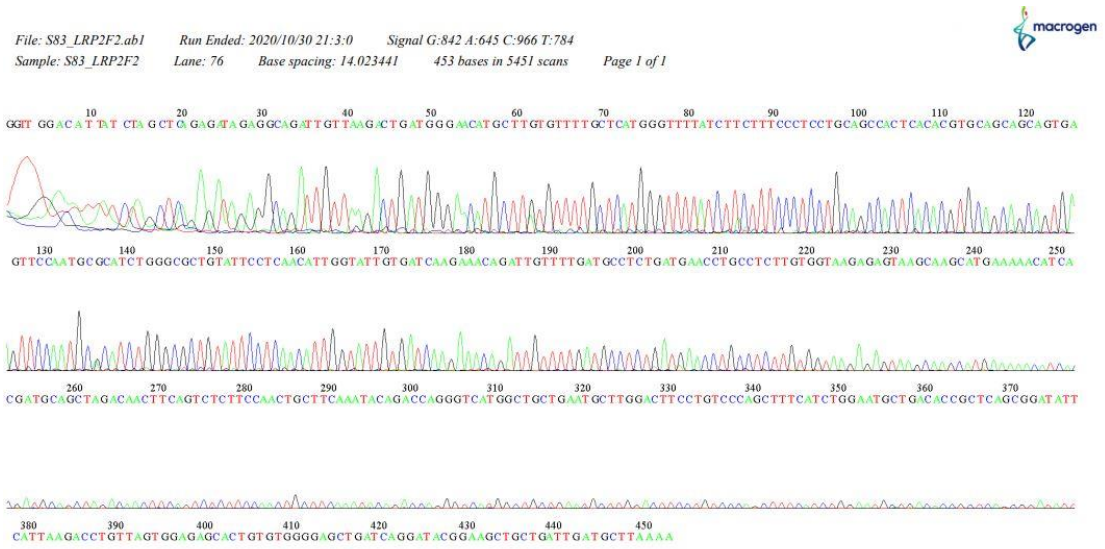


Figure 100 Chromatogram of sequencing from PCR-LRP2 sample 69

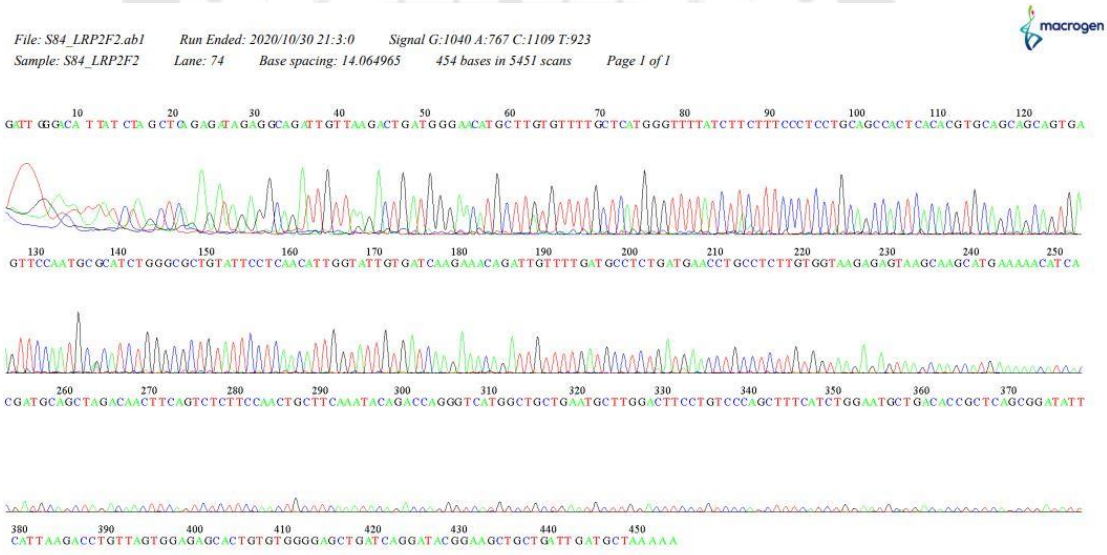


Figure 101 Chromatogram of sequencing from PCR-LRP2 sample 70

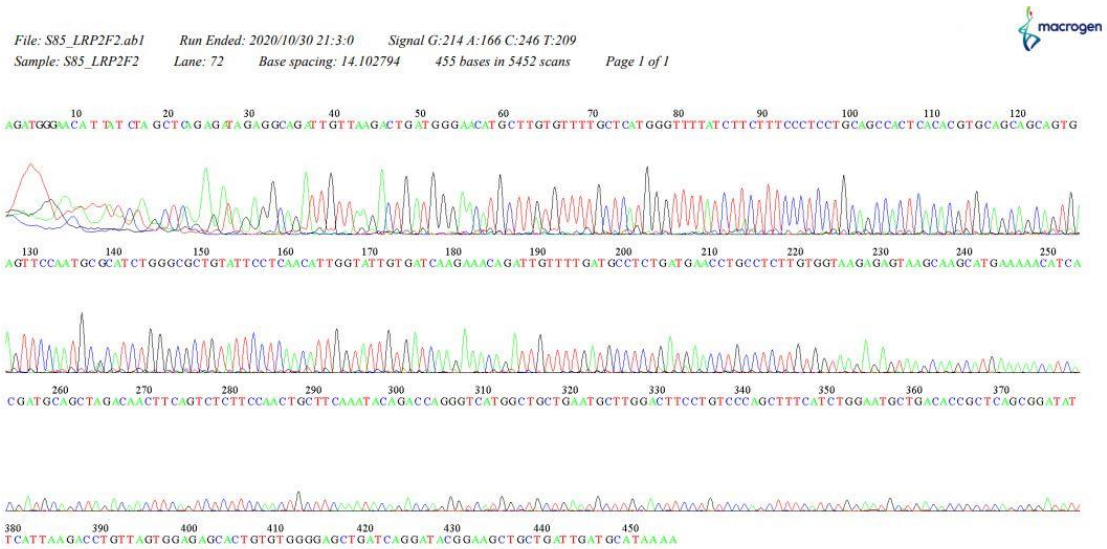


Figure 102 Chromatogram of sequencing from PCR-LRP2 sample 71

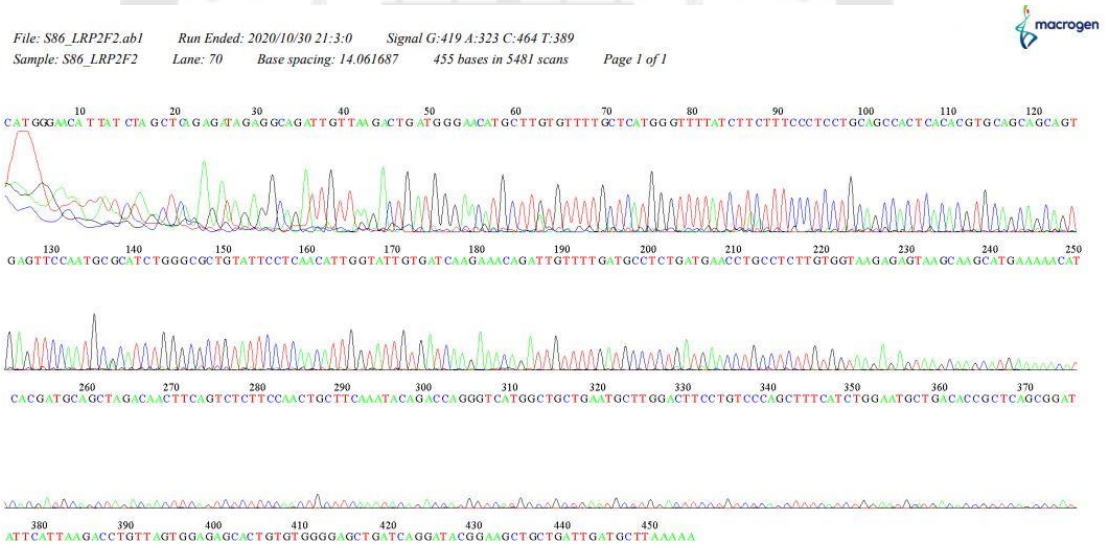


Figure 103 Chromatogram of sequencing from PCR-LRP2 sample 72

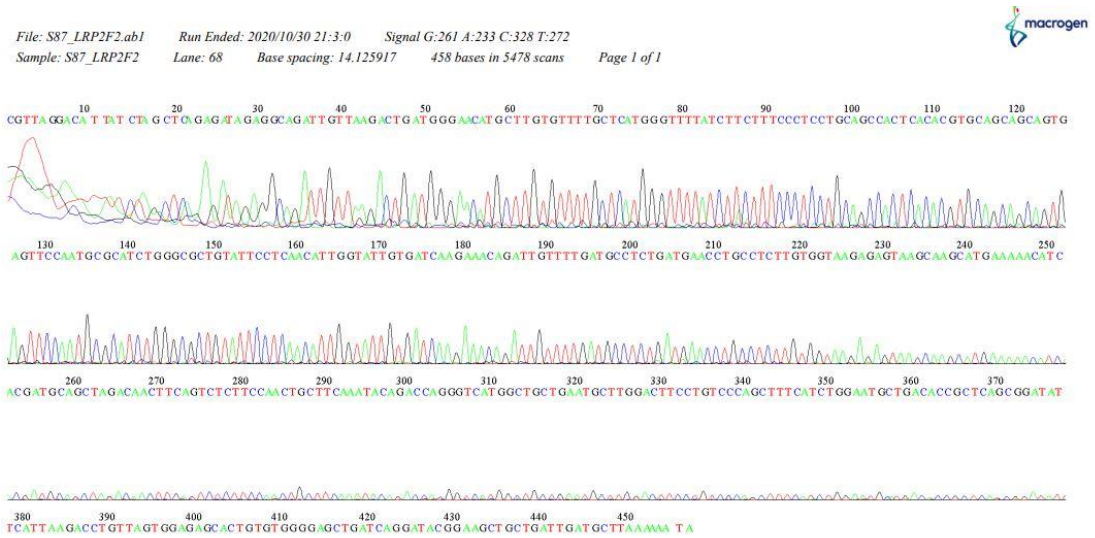


Figure 104 Chromatogram of sequencing from PCR-LRP2 sample 73

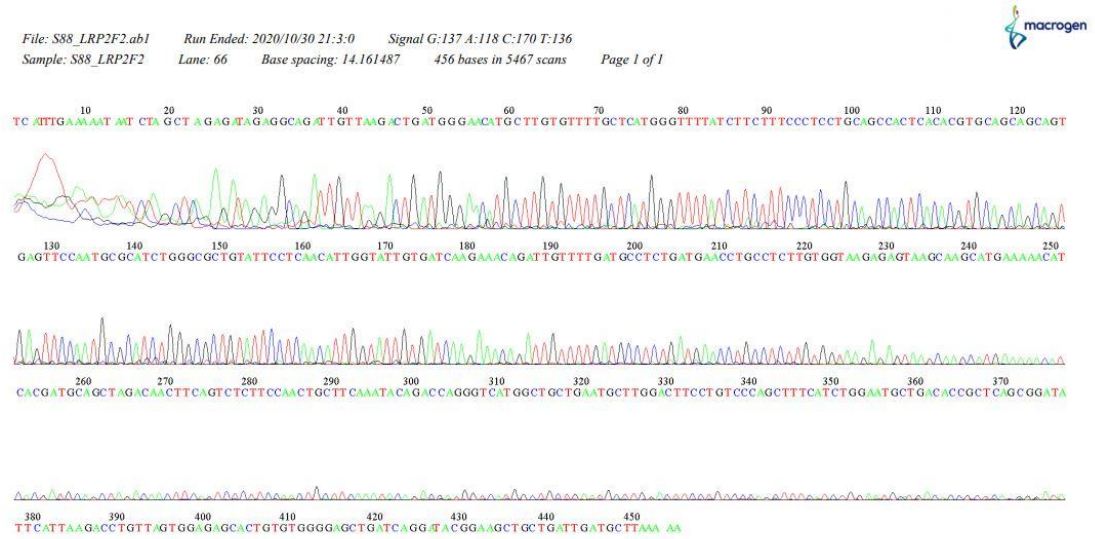


Figure 105 Chromatogram of sequencing from PCR-LRP2 sample 74

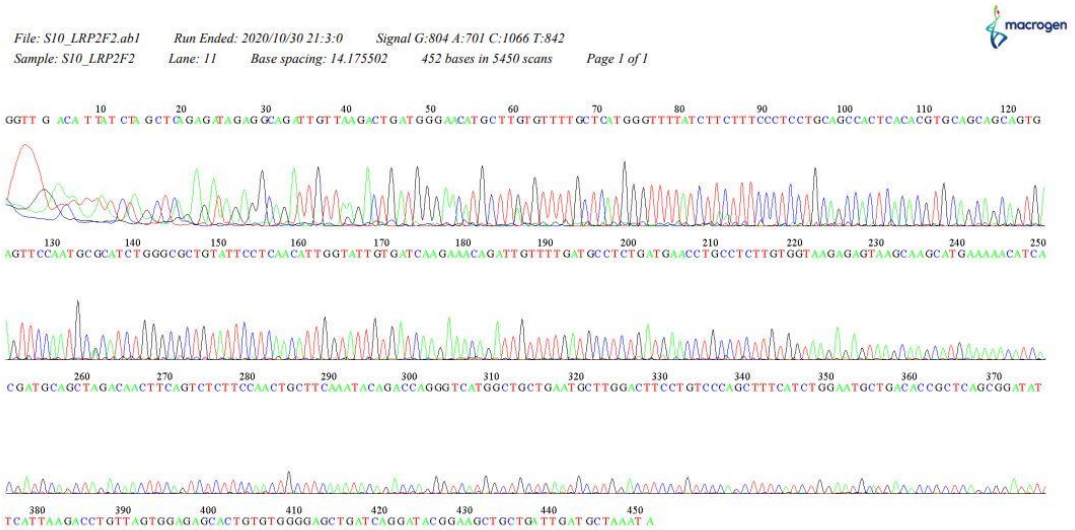


Figure 106 Chromatogram of sequencing from PCR-LRP2 sample 75

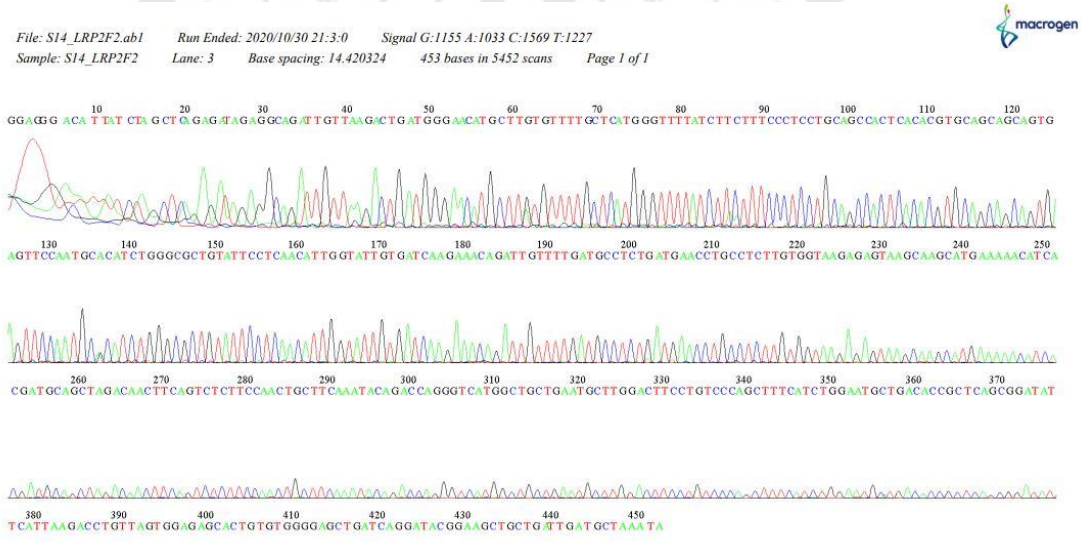


Figure 107 Chromatogram of sequencing from PCR-LRP2 sample 76

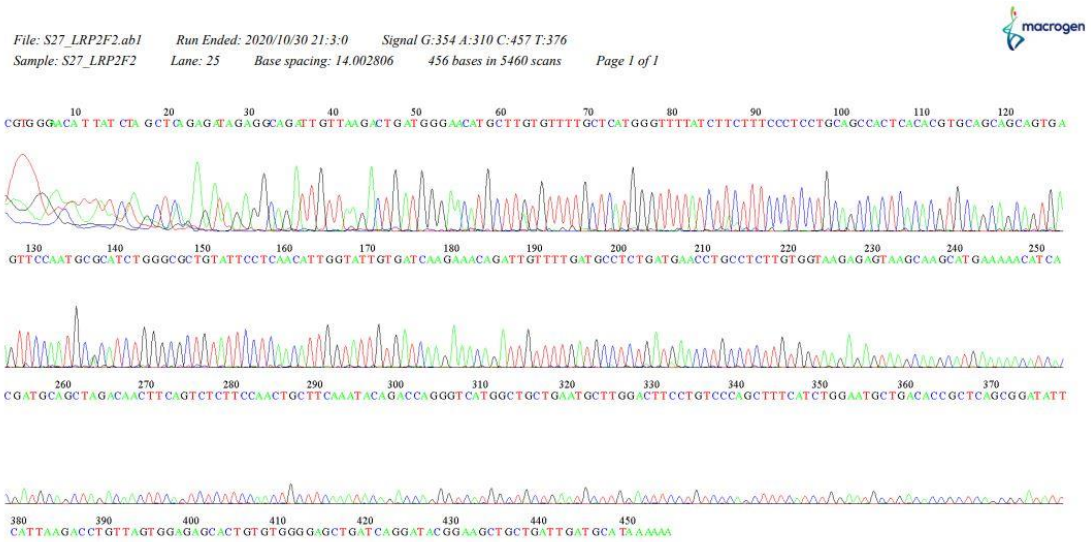


Figure 108 Chromatogram of sequencing from PCR-LRP2 sample 77

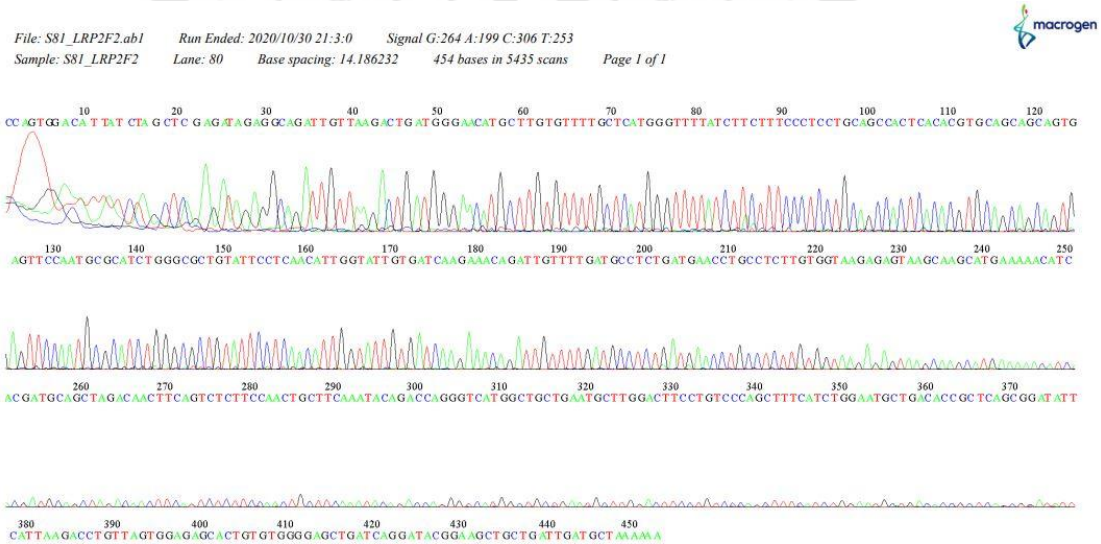


Figure 109 Chromatogram of sequencing from PCR-LRP2 sample 78

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