Research Journal of Medical and Public Health 6 (1)

Diagnostic Test for Pyruvate Kinase Deficiency (PKD)

Bibi Fawziyya Mohabeer

Technological University Dublin (Tud)

Abstract

Pyruvate Kinase deficiency (PKD) is the most common enzyme defect that interrupts the glycolytic pathway of the Red blood cell. Pyruvate Kinase is an important enzyme which allows the production of an ATP molecule in the final phase of the glycolytic pathway. The four types of PK enzymes are M1, M2, L and R. The isozymes L and R are encoded by the same gene known as PKLR gene. A mutation in this gene causes PKD, an inherited disease (which is autosomal recessive) that influences the RBC and most commonly cause haemolytic anaemia. The PKLR gene is located on chromosome 1q21. There are various mutations that can occur on the PKLR gene. However, this report focuses on the diagno stic test for the mutation on exon 6 of PKLR gene. The mutation on exon 6 leads to less restriction site (using enzyme Hyp188III), thus alters the number and sizes of PCR product obtained.

Keywords: *Pyruvate, Kinase, PKD, PKLR gene, chromosome 1q21, exon 6, primers, restriction site, Hyp188III, PCR product.*

1.0 Introduction

Pyruvate Kinase Deficiency (PKD)

Pyruvate kinase deficiency (PKD) is the most common enzyme defect that disturbs the glycolytic pathway of the Red Blood Cells (RBC) (OMIM 266200, 2016). There are 4 types of PK isozymes that exist in humans (M1, M2, L and R)- both PK-L is located in the liver, kidney and gut while PK-R available in RBC. The same gene encodes for the L and R form of isozymes- The gene is known as PKLR.

Pyruvate Kinase (PK) is the enzyme needed in the final phase of the glycolytic pathway, in which an ATP molecule is produced (The comprehensive Enzyme information system, 2017). Type 2 diabetes has strongly associated with genetic components. Pyruvate kinase (PK) is an ubiquitously expressed enzyme that work as a catalyst in the formation of pyruvate from phosphoenolpyruvate; and generate ATP simultaneously (The comprehensive Enzyme information system, 2017).

In liver, the PK-L isoform is expressed in pancreatic Beta cells, small intestine and proximal renal tubule (Yamada and Noguchi, 1999). The gene is upregulated by glucose through the carbohydrate response element in the L-type promoter region (Yamada and Noguchi, 1999) and is among the downstream of hepatocyte nuclear factor (HNF)-1α (Wang *et al*., 2000; Shih *et al*., 2001). PK-L shares the issue distribution of genes known to cause more severe early-onset diabetes which is referred as MODY, Maturity Onset Diabetes of the Young (Fajans *et al*., 2001).

Pyruvate kinase deficiency (PKD) is an inherited disease that influences the RBC, which transport oxygen to tissues of other parts in the body; People with this disorder have a medical condition called chronic haemolytic anaemia, in which RBC is lysed (undergo haemolysis) prematurely, resulting in a dearth of erythrocytes and thus causing anaemia (Genetic Home Reference, 2017). Specifically, pyruvate kinase deficiency is a common cause of a type of inherited haemolytic anemia. In the inherited disease nonspherocytic haemolytic anemia, the erythrocytes do not adhere to its spherical shape as they do in other types of haemolytic anaemia.

Signs and Symptoms

Chronic haemolytic anaemia can show several signs and symptoms such as:

- pale skin (pallor)
- jaundice
- fatigue
- dyspnea
- a rapid heart rates

Other common signs and symptoms (Genetic Home Reference, 2017). include:

- Splenomegaly (the term used for an enlarged spleen)
- an excess of iron present in the blood
- gallstones

Among patients who suffer from PKD, haemolytic anaemia and associated complications may range from mild to severe. Some patient can show few or no symptoms. Severity of the condition varies from case to case- It can be life-threatening in infancy, and these individuals would need to have regular blood transfusions to survive. The symptoms of this disorder may deteriorate if there is an infection or during pregnancy (Genetic Home Reference, 2017).

Cause of PKD

Pyruvate kinase deficiency is the most common type inherited disease that cause of nonspherocytic haemolytic anemia. Over 500 families are found to be affected, and previous studies revealed that this disorder might remain underdiagnosed in some patient since mild cases would not reveal appropriate signs and symptoms that lead to this diagnosis (Genetic Home Reference, 2017). This is because of the pattern that this disease is inherited from the parent. Carriers would not be affected.

Pyruvate kinase deficiency is caused by mutations the PKLR gene. The PKLR gene is active in the liver and in red blood cells, where it provides information to generate an enzyme called pyruvate kinase (Genetic Home Reference, 2017). However, Pyruvate kinase deficiency may also happen as a complication of a different blood diseases, for example leukaemia. These cases are known as secondary PKD and thus are not genetically inherited disease.

Genetic Inheritance

This condition is inherited in an autosomal recessive pattern. This means both copies of the gene in each cell (one from maternal and the other from paternal) have mutations (Genetic Home Reference, 2017). The parents of an individual with an autosomal recessive condition each carry one copy of the mutated gene and one copy of the normal gene; and they are the carrier (not affected). Hence, they do not usually reveal signs and symptoms of this condition.

PKLR gene

The PKLR gene (which is over 9·5 kb) is located on chromosome 1q21 (Figure 1) (Satoh *et al*, 1988) where it directs the transcription which is specific for the liver (L-PK) and the RBC (R-PK) isoenzymes by using their promoters alternately (Noguchi *et al*, 1987; Kanno *et al*, 1992).

The coding sequences is divided into 12 exons. 10 of them are shared by the two forms L & R, whereas the exons 1 and 2 are precise for the RBC and the hepatic isoenzyme, correspondingly (Noguchi *et al*, 1987; Tani *et al*, 1988; Kanno *et al*, 1991). The cDNA that codes RPK is made up of 2060 base pairs and they code for 574 amino acids (Kanno *et al*, 1991). The R-type promoter region of the PKLR gene, located in the 5′ flanking region- upstream from the first exon; two CAC boxes and four GATA motifs have been recognized in the 270 base pairs from the translational start codon. The proximal 120-base pairs region has a basal promoter activity. In addition to that, the region from −120 to −270 act as a powerful enhancer in erythroid cells (Kanno *et al*, 1992).

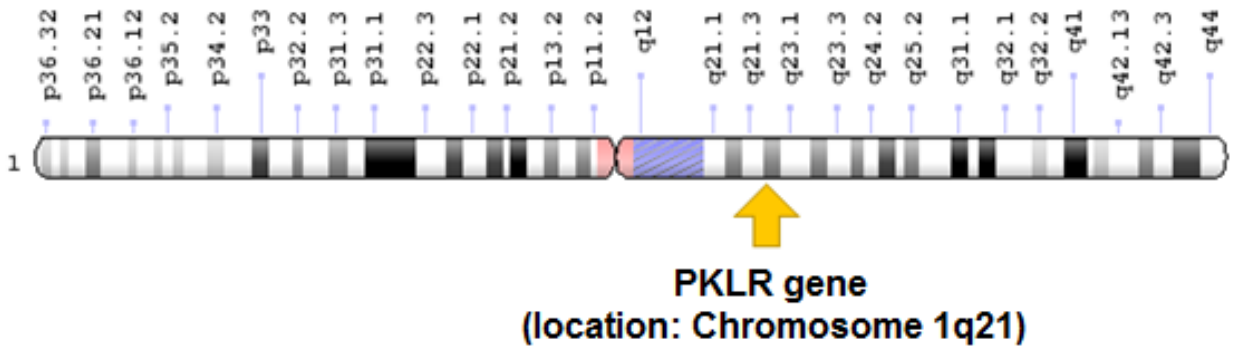


Figure 1: PKLR gene on chromosome 1.
Source: (Genetic Home Reference, 2017*).*

Common mutations of PKLR

Over hundreds of mutations are associated with non-spherocytic haemolytic anaemia (Table 1). About eight polymorphic sites (Table 2) have been described in the PKLR gene (Bianchi and Zanella, 2000). Mutations and polymorphisms are usually designated using the RPK cDNA sequence of the PKLR gene, with the A of the initiation ATG being assigned number +1 (GenBank accession numbers D10326 and D904655). The GenBank accession numbers are U47654 for the genomic DNA and D13232 for the putative promoter region.

Table 1: Mutations in the *PK-LR* gene associated with congenital non-spherocytic haemolytic anaemia reported in the literature.

cDNA nucleotide substitution	Effect	Exon(s)	Reference
−83g→c		Promoter	van Wijk *et al* (2003)
−72a→g			Manco *et al* (2000)
IVS2(-1) g→a	Splice Site	IVS2	Lenzner *et al* (1994a)
107 C→G	36 Ala→Gly	3	Fermo *et al* (2005)
110 G→A	37 Gly→Gln	3	Beutler *et al* (1997)
183 16 bp 184 ins	Frameshift	3	Kugler *et al* (2000)
227–231 TGGAC del	Frameshift	3	Zanella *et al* (1997)
238 T→C	80 Ser→Pro	3	Uenaka *et al* (1995)

cDNA nucleotide substitution	Effect	Exon(s)	Reference
244C del	Frameshift	3	Fermo *et al* (2005)
269 T→A	90 Ile→Asn	3	van Solinge *et al* (1997b)
278 C→T	93 Thr→Ile	3	Fermo *et al* (2005)
283 G→A	95 Gly→Arg	3	van Solinge *et al* (1997b)
IVS3(-2) a→c	Splice Site	IVS3	Zanella *et al* (1997)
IVS3(-2) a→t	Splice Site	IVS3	Kanno *et al* (1997)
5006 bp (IVS3→nt 1431) del	ex 4–11 del	IVS3-ex10	Fermo *et al* (2005)
ex 4–10 del	–	4–10	Costa *et al* (2005)
307 C del	Frameshift	4	Baronciani and Beutler (1995)
320 T→C	107 Met→Thr	4	Baronciani *et al* (1995a)
331 G→A	111 Gly→Arg	4	van Solinge *et al* (1997b)
343 G→C	115 Ala→Pro	4	Rouger *et al* (1996b)
C 346–349del→C 346 insAACATTG	Arg Leu 116→Gln His Cys	4	Pissard *et al* (1999)
359 C→T	120 Ser→Phe	4	Rouger *et al* (1996b)
389 C→A	130 Ser→Tyr	5	Cohen-Solal *et al* (1998)
391–393 ATC del	131 Ile del	5	Baronciani and Beutler (1993)
401 T→A	134 Val→Asp	5	Baronciani and Beutler (1993)
403 C→T	135 Arg→Trp	5	Fermo *et al* (2005)

cDNA nucleotide substitution	Effect	Exon(s)	Reference
409 G→A	137 Ala→Thr	5	Fermo *et al* (2005)
434 C del	Frameshift	5	Kanno *et al* (1994c)
458T→C	153 Ile→Thr	5	Kugler *et al* (2000)
464 T→C	155 Leu→Pro	5	Baronciani and Beutler (1993)
476 G→T	159 Gly→Val	5	Demina *et al* (1998)
487 C→T	163 Arg→Cys	5	Neubauer *et al* (1991)
507 G→A	Splice Site	5	Fermo *et al* (2005)
IVS5(+1) g→a	Splice Site	IVS5	van Wijk *et al* (2004)
514 G→C	172 Glu→Gln	6	Zanella *et al* (1997)
601 C→T	201 Trp→Arg	6	Pissard *et al* (1999)
603 G→A	201 Trp→End	6	Baronciani *et al* (1995a)
628–629 GT del	Frameshift	6	Lenzner *et al* (1997a)
656 T→C	219 Ile→Thr	6	Kugler *et al* (2000)
661 G→A	221 Asp→Asn	6	Fermo *et al* (2005)
663 GAC 664 ins	221 Asp 222 ins	6	Kanno *et al* (1994c)
671 T→C	224Ile→Thr	6	Pissard *et al* (1999)
IVS6(-2) a→t	Splice Site	IVS6	Zanella *et al* (2001a)
721 G→T	241 Glu→End	7	Baronciani and Beutler (1993)

cDNA nucleotide substitution	Effect	Exon(s)	Reference
757 A→G	253 Asn→Asp	7	van Wijk *et al* (2001)
787 G→A	263 Gly→Arg	7	Lenzner *et al* (1997a)
787 G→T	263 Gly→Trp	7	Zanella *et al* (1997)
808 C→T	270 Arg→End	7	Baronciani and Beutler (1995)
814 C→G	272 Leu→Val	7	van Wijk *et al* (2001)
823 G→C	275 Gly→Arg	7	Baronciani *et al* (1995a)
823 G→A	275 Gly→Arg	7	Zanella *et al* (1997)
841 G→A	281 Asp→Asn	7	Kanno *et al* (1994c)
859 T→G	287 Phe→Val	7	Kanno *et al* (1994c)
859 T→C	287 Phe→Leu	7	Fermo *et al* (2005)
862 G→T	288Val→Leu	7	Aizawa *et al* (2003)
877 G→A	293Asp→Asn	7	Kugler *et al* (2000)
884 C→T	295 Ala→Val	7	Demina *et al* (1998)
929 T→A	310 Ile→Asn	7	van Solinge *et al* (1996)
941 T→C	314 Ile→Thr	7	Kanno *et al* (1994a)
943 G→A	315 Glu→Lys	7	Demina *et al* (1998)
948 C→G	316 Asn→Lys	7	Costa *et al* (2005)
958 G→A	320 Val→Met	7	Fermo *et al* (2005)

cDNA nucleotide substitution	Effect	Exon(s)	Reference
IVS7(+1) g→t	Splice Site	IVS7	Kanno *et al* (1997)
991 G→A	331 Asp→Asn	8	Kugler *et al* (2000)
993 C→A	331 Asp→Glu	8	Baronciani and Beutler (1995)
994 G→A	332 Gly→Ser	8	Lenzner *et al* (1994a)
1003 G→A	335 Val→Met	8	Zanella *et al* (2001b)
1006 G→T	336 Ala→Ser	8	Lenzner *et al* (1994a)
1010 G del	Frameshift	8	Cotton *et al* (2001)
1010 G→C	337 Arg→Pro	8	Pastore *et al* (1998)
1010 G→A	337 Arg→Gln	8	Lenzner *et al* (1997a)
1015 G→C	339 Asp→Gln	8	Zarza *et al* (1998)
1022 G→C	341 Gly→Ala	8	Baronciani and Beutler (1995)
1022 G→A	341 Gly→Asp	8	Demina *et al* (1998)
1024 A→T	342 Ile→Phe	8	Layton *et al* (1996)
1042–1044 AAG del	348 Lys del	8	Zanella *et al* (2001b)
1044 G→T	348 Lys→Asn	8	Kanno *et al* (1997)
1055 C→A	352 Ala→Asp	8	Kugler *et al* (2000)
1060–1062 AAG del	354 Lys del	8	Lenzner *et al* (1994a)
1070 T→C	357 Ile→Thr	8	Zarza *et al* (1998)

cDNA nucleotide substitution	Effect	Exon(s)	Reference
1073 G→A	358 Gly→Glu	8	van Wijk *et al* (2001)
1075 C→T	359 Arg→Cys	8	Kanno *et al* (1994c)
1076 G→A	359 Arg→His	8	Baronciani and Beutler (1993)
1081 A→G	361 Asn→Asp	8	Lenzner *et al* (1994a)
1089 G 1090 ins	Frameshift	8	Baronciani and Beutler (1995)
1091 G→A	364 Gly→Asp	8	van Solinge *et al* (1997b)
1094 A→T	365 Lys→Met	8	Fermo *et al* (2005)
1102 G→T	368 Val→Phe	8	Kanno *et al* (1993a)
IVS8(+2) t→g	Splice site	IVS8	Manco *et al* (1999)
1121 T→C	374 Leu→Pro	9	van Wijk *et al* (2001)
1127 G→T	376 Ser→Ile	9	Lenzner *et al* (1997a)
1151 C→T	384 Thr→Met	9	Neubauer *et al* (1991)
1153 A→T	385 Arg→Trp	9	Beutler and Gelbart (2000)
1154 G→A	385 Arg→Lys	9	van Wijk *et al* (2001)
1160 A→G	387 Glu→Gly	9	Zanella *et al* (2001b)
1168 G→A	390 Asp→Asn	9	Zanella *et al* (1997)
1174 G→A	392 Ala→Thr	9	Lenzner *et al* (1994a)
1178 A→G	393 Asn→Ser	9	Baronciani and Beutler (1995)

cDNA nucleotide substitution	Effect	Exon(s)	Reference
1179 T→A	393 Asn→Lys	9	Baronciani and Beutler (1995)
1181 C→A	394 Ala→Asp	9	Zanella *et al* (2001b)
1181 C→T	394 Ala→Val	9	Zanella *et al* (2001b)
1190 A→T	397 Asp→Val	9	Fermo *et al* (2005)
1193 G→C	398Gly→Ala	9	Pissard *et al* (1999)
1195 G del	Frameshift	9	Rouger *et al* (1996a)
1203 AGC 1204 ins	401 Ser 402 ins	9	Lenzner *et al* (1994a)
1209 G→A	403 Met→Ile	9	Fermo *et al* (2005)
1223 C→T	408 Thr→Ile	9	Zarza *et al* (1998)
1228A→G	410Lys→Glu	9	Pissard *et al* (1999)
1231G→A	411Gly→Ser	9	Park-Hah *et al* (2005)
1232 G→C	411 Gly→Ala	9	Fermo *et al* (2005)
1261 C→A	421 Gln→Lys	9	Kanno *et al* (1992b)
1269 G→A	Splice Site	9	Kanno *et al* (1994c)
1269 G→C	Splice Site	9	Zanella *et al* (1997)
Ivs9(+43)c→t	Splice site	IVS9	Fermo *et al* (2005)
Ivs9(-1)g→c	Splice site	IVS9	Fermo *et al* (2005)
1276 C→T	426 Arg→Trp	10	Kanno *et al* (1994c)

cDNA nucleotide substitution	Effect	Exon(s)	Reference
1277 G→A	426 Arg→Gln	10	Kanno *et al* (1993b)
1281 G→T	427 Glu→Asp	10	Lenzner *et al* (1997a)
1291 G→A	431 Ala→Thr	10	Zarza *et al* (1998)
1318 G→T	440 Glu→End	10	Sedano *et al* (2004)
1369 A→G	457 Ile→Val	10	Fermo *et al* (2005)
1373 G→A	458 Gly→Asp	10	Baronciani and Beutler (1995)
1376 C→T	459 Ala→Val	10	Baronciani *et al* (1995a)
1378 G→A	460 Val→Met	10	Baronciani and Beutler (1995)
1403 C→T	468 Ala→Val	10	Kanno *et al* (1994a)
1409C→A	470Ala→Asp	10	Pissard *et al* (1999)
1436 G→A	479 Arg→His and Splice site	10	Kanno *et al* (1994a)
Ivs 10(+1) g→c	Splice site	IVS10	Manco *et al* (1999)
1437–1618 del	Frameshift	11	Baronciani and Beutler (1995)
1454 C→T	485 Ser→Phe	11	Lenzner *et al* (1997a)
1456 C→T	486 Arg→Trp	11	Baronciani and Beutler (1993)
1462 C→T	488 Arg→End	11	van Solinge *et al* (1997b)
1468 C→T	490 Arg→Trp	11	Kanno *et al* (1994c)
1483 G→A	495 Ala→Thr	11	Kugler *et al* (2000)

cDNA nucleotide substitution	Effect	Exon(s)	Reference
1484 C→T	495 Ala→Val	11	Baronciani and Beutler (1993)
1488 C del	Frameshift	11	Rouger *et al* (1996b)
1492 C→T	498 Arg→Cys	11	van Solinge *et al* (1997b)
1493 G→A	498 Arg→His	11	Lenzner *et al* (1994a)
1501 C→T	501 Gln→End	11	Baronciani *et al* (1995a)
1508 C→T	503 Ala→Val	11	Zarza *et al* (1999)
1511 G→T	504 Arg→Leu	11	Demina *et al* (1998)
1515–1518dupl	Frameshift	11	Zanella *et al* (2001b)
1516 G→A	506 Val→Ile	11	Zarza *et al* (2000)
1523 T→G	508 Leu→End	11	Pastore *et al* (1998)
1528 C→T	510 Arg→End	11	Demina *et al* (1998)
1529 G→A	510 Arg→Gln	11	Baronciani and Beutler (1993)
1552 C→A	518 Arg→Ser and Splice Site	11	Zanella *et al* (1997)
1574 G 1575 ins	Frameshift	11	Baronciani *et al* (1995a)
1594 C→T	532 Arg→Trp	11	Lakomek *et al* (1994)
1595 G→A	532 Arg→Gln	11	Zarza *et al* (1998)
1618 Ivs11(+1) g Del	Splice Site	IVS11	van Wijk *et al* (2001)
Ivs11(-3) c→g	Splice Site	IVS11	van Wijk *et al* (2001)

cDNA nucleotide substitution	Effect	Exon(s)	Reference
1654 G→A	552 Val→Met	12	Baronciani *et al* (1995a)
1670 G→C	557 Gly→Ala	12	Manco *et al* (1999)
1675 C→G	559 Arg→Gly	12	Baronciani *et al* (1995a)
1675 C→T	559 Arg→End	12	Zarza *et al* (1998)
1698 C→A	566 Asn→Lys	12	Kanno *et al* (1994c)
1706 G→A	569 Arg→Gln	12	van Wijk *et al* (2001)

Source: Bianchi and Zanella (2000).

Table 2: Polymorphisms reported in the *PK-LR* gene.

Polymorphic Site CDNA Number	Polymorphic site genomic number	Exon	Reference
. *T-stretch occurring in the two forms $(T)_{10}$ and $(T)_{19}$.			
−342 T/A		Promoter	van Wijk *et al* (2003)
−248T del			van Wijk *et al* (2003)
IVS5(+51)C/T	2838 C/T	IVS5	Baronciani *et al* (1995b)
$T_{10/19}$*	5972–5981 (T_{10})	IVS10	Lenzner *et al* (1997b)
Microsatellite ATT	7181–7222 (14 ATT)	IVS11	Lenzner *et al* (1994b)
1705 A/C	7619 A/C	12	Kanno *et al* (1992b)
1738 C/T	7652 C/T	12	Zanella *et al* (1997)
1992 C/T	7906 C/T	12	Lenzner *et al* (1994b)

Source: Bianchi and Zanella (2000).

There are three types of mutations that are very common in patients with PKD and these are: 1529A, 1456T and 1468T. These variants are strongly associated with ethnic and regional background. The 1529A is the most common mutation in the USA (45%) (Baronciani & Beutler, 1995) and in Northern and Central Europe (41%) (Lenzner *et al*, 1997). 1456T is most common in southern Europe (32% in Spain, 35% in Portugal and 29% in Italy). However, mutation 1529A is infrequent (Demina *et al*, 1998; Zarza *et al*, 1998). 1468T is very common in Asia (9/16 nonrelated family) (Kanno *et al*, 1994). Other mutations exist less frequently in White people (Zarza *et al*, 1998; Fermo *et al*, 2005).

Treatments for PKD

Different types of drugs and chemicals administered to enchance *in vivo* activity (Zanella *et al*, 1976). There is no specific therapy of treatment available for PK deficiency. The treatment of this disease is hence sustained as a supporting technique for the better health of individuals. Red cell transfusions may be required in severely anaemic cases- especially in the first years of life; the haemoglobin then have a tendency to stabilize. Then transfusions are no longer necessary unless the anaemia is aggravated by intercurrent infections, pregnancy or other circumstances (Dacie, 1985). As the delivery of oxygen to tissues is highly efficient because of the high 2,3-DPG content, the decision to transfuse a PK-deficient patient is based on the clinical condition rather than the haemoglobin level.

2.0 Diagnostic test for PKD

PKLR mutation- Exon 6

The specific mutation that has been considered in this report was a mutation on the exon 6 of the gene PKLR. The sequence of this exon was accessed using the accession number for genomic sequence (GenBank U47654) (Pissard *et al.*, 2006).

Location	n° cDNA	n° genomic	Hypothetical consequences on mRNA processing
(B) New mutations affecting a splice site			
IVS 1 (D)	c.100 + 10 G > A	nt g. 110 G > A	Alteration of a splice enhancer (McCullough & Berget, 1997)
IVS 4 (D)	c.375 + 2 T > C	nt g. 5721 T > C	Destruction of a donor splice site, addition of 32 aa in protein chain
IVS 4 (D)	c.375 + 10 G > T	nt g. 5721 G > T	Alteration of a splice enhancer (McCullough & Berget, 1997)
IVS 4 (A)	c.376 − 1 G > A	nt g. 5818 G > A	Destruction of an acceptor splice site
Exon 6 (D)	c.694 G > T	nt g. 6270 G > T	Gly232Cys and IVS 6–1 G > T, alteration of a donor splice site and 'stop' 85 codons downstream
IVS 9 (D)	c.1269 + 5 G > A	nt g. 7948 G > A	Alteration of a donor splice site, addition of 31 AA, FS in exon 10 with a 'stop' 15 codons downstream
Exon 11 (D)	c.1618 G > C	nt g. 9630 G > C	Gly540Arg and IVS11–1 G > C alteration of a donor splice site, 'stop' 68 codons downstream

The mutations in the *PK-LR* gene are designated according to the numbering system, with nucleotide numbering starting at position 40, which is the A of the initiation codon in the reference sequence (GenBank D13026). The splice mutations are also numbered using genomic sequence (GenBank U47654) (Bianchi & Zanella, 2000).
*Mutations reported by Pissard *et al* (1999) are in italics.

Figure 2: Mutation in Exon 6
Source: Pissard et al., 2006.

Consequences of Mutated Exon 6

A mutation on exon 6- G > T.

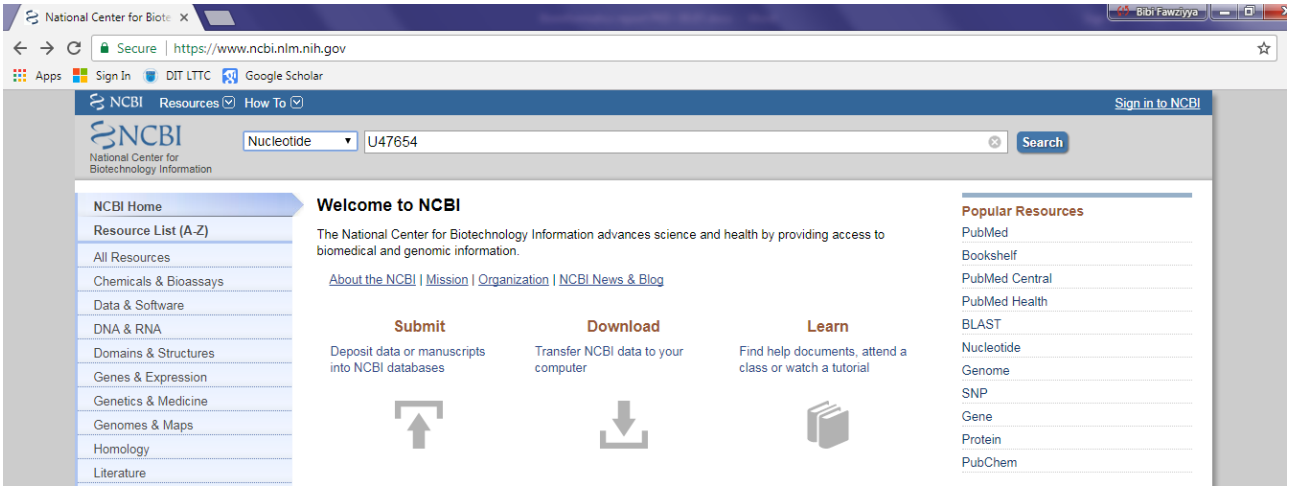
Gly 232 Cys, and IVS 6-1 G > T, alteration of a donar splice site and `stop` 85 codons downstream.

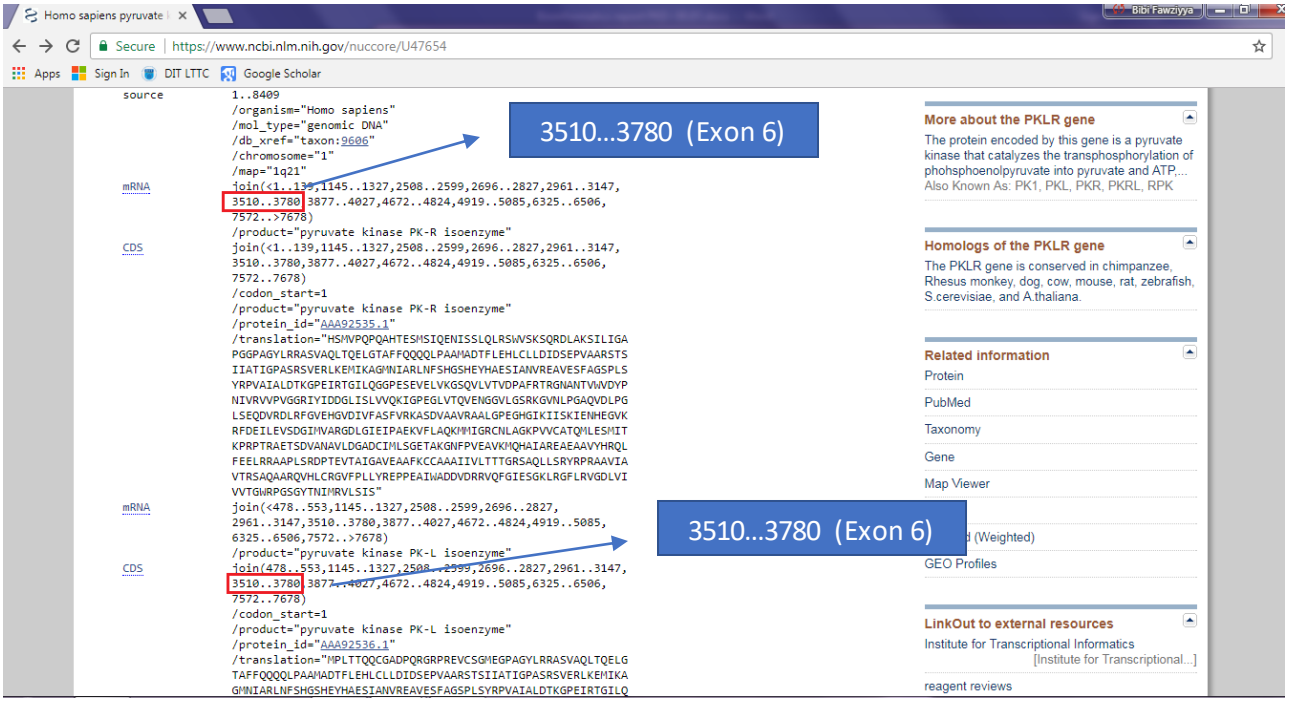
Diagnosis of PKLR mutation- Exon 6

The diagnosis of this specific mutation was done following a step by step process.

STEP 1:

The test was first started by retrieving the sequence of exon 6 of the gene PKLR. This was done using the accession number (GenBank U47654) in NCBI (Figure 3). Exon 6 is from 3510 to 3780, however, extra 100 was included in the test (with due consideration to find primer sets further on in the diagnostic test). Hence, sequence included was 3510 to 3880 in Bioedit.





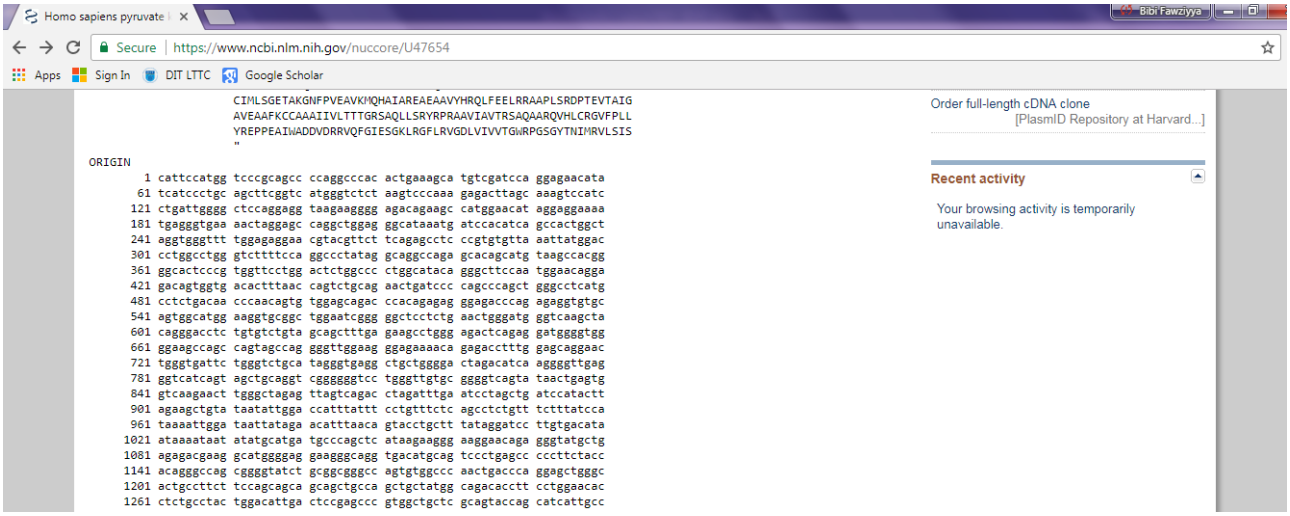
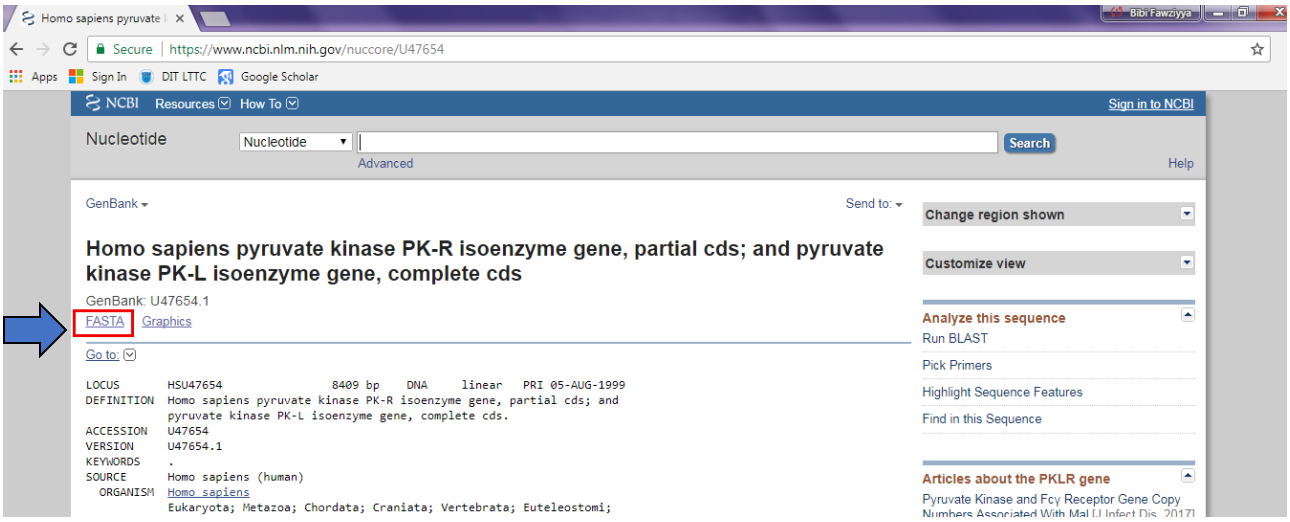


Figure 3: Accessing PKLR gene using accession number U47654.

STEP 2:

The original sequence contains numbers in the left margin, which is not appropriate to use while performing a diagnosis. Therefore, it was essential to get the sequences in the FASTA version. Click on FASTA.



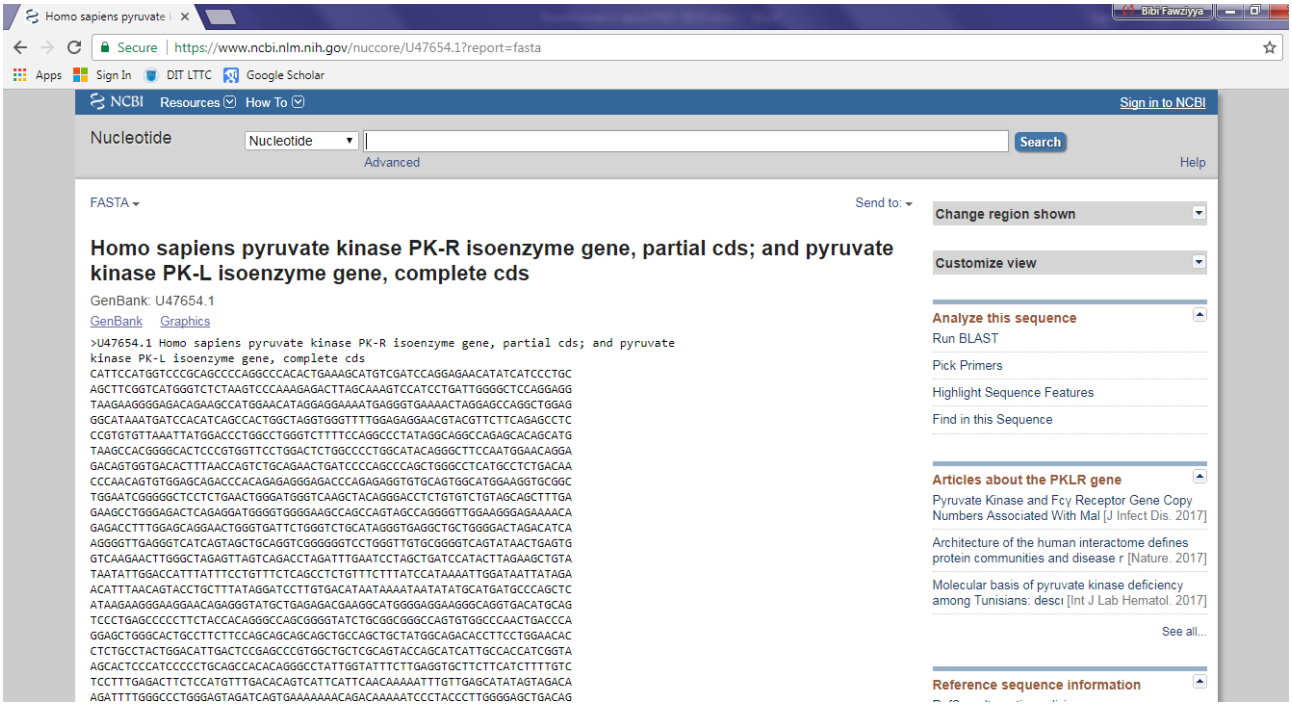
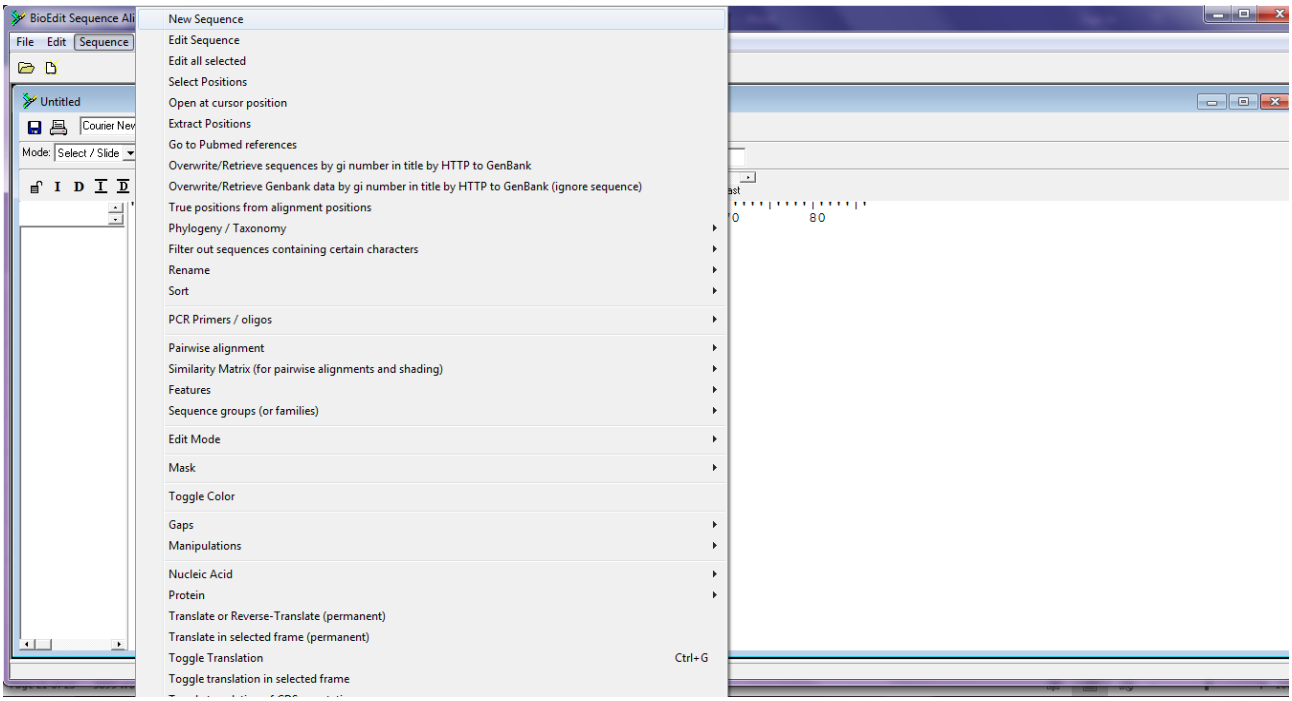


Figure 4: Getting the Sequence in FASTA version.

STEP 3:

- Get the FASTA sequence > Copy > and Paste into Bio Edit
- Open Bioedit, go to sequence > New sequence > Edit sequence to obtain exon 6 (3510 to 3880) > Save the sequence as PKLR_Exon 6



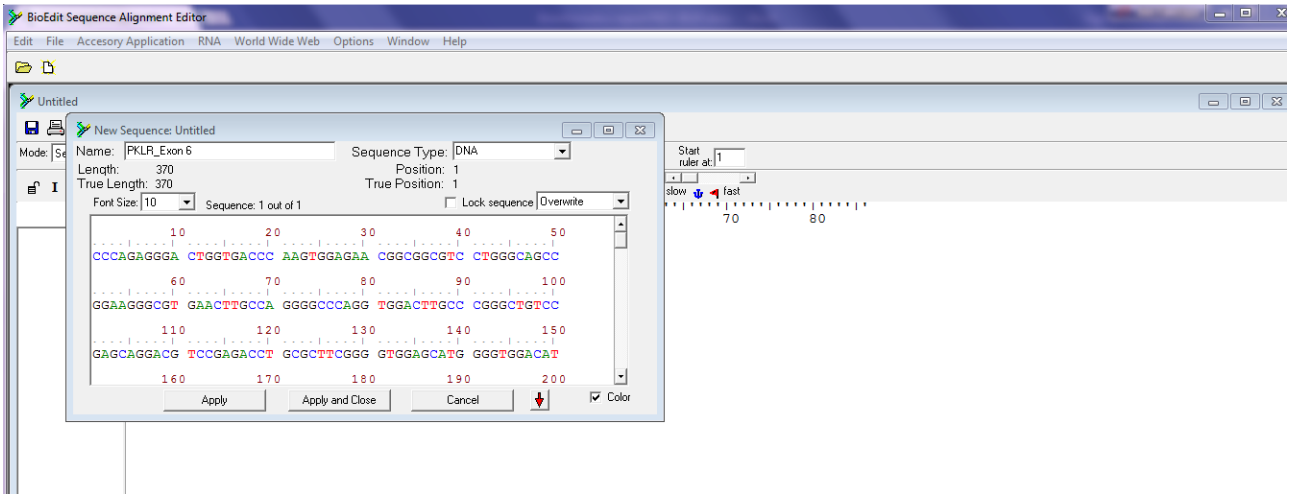


Figure 5: Getting the Exon 6 into bioedit.

The same step is repeated to get the mutated sequence and Edit the sequence at the location of mutation (which is G > T at 233). Mutated sequence was saved as PKLR*_Exon 6.

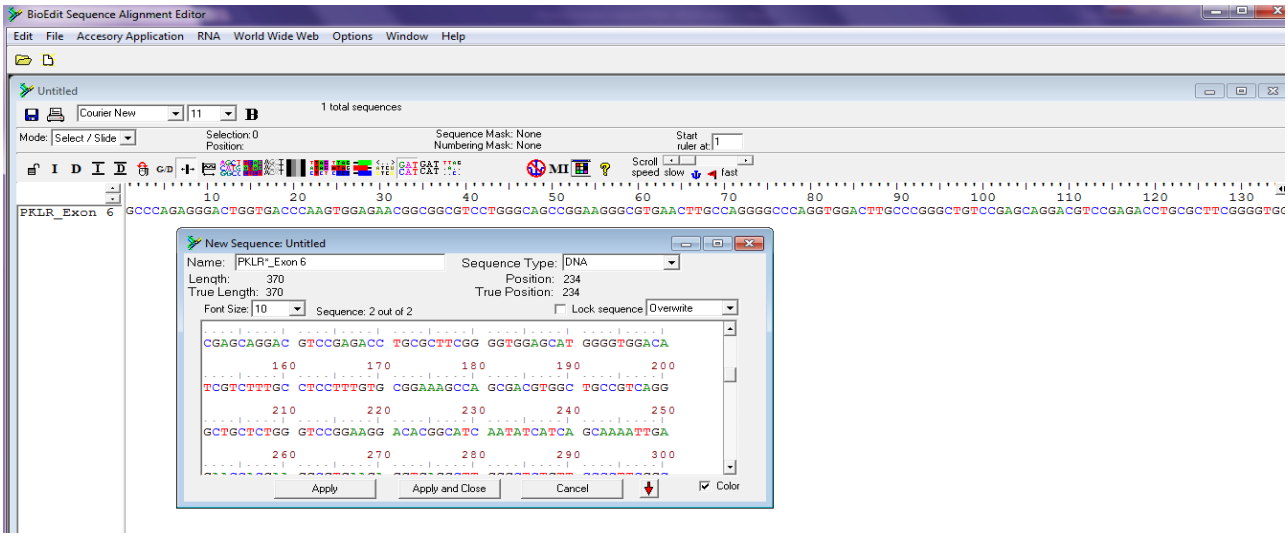


Figure 6: Getting the mutated Exon 6.

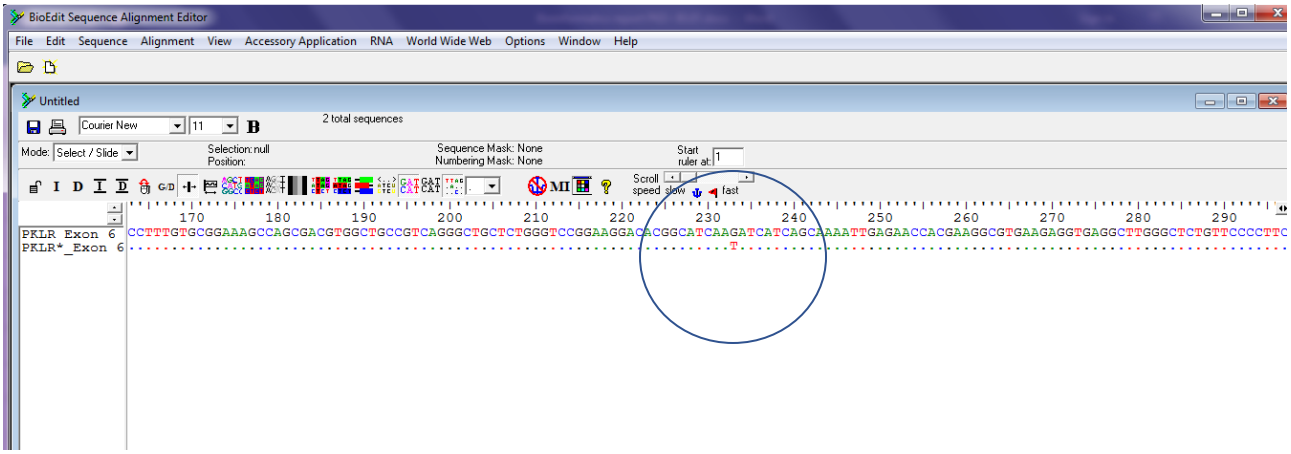
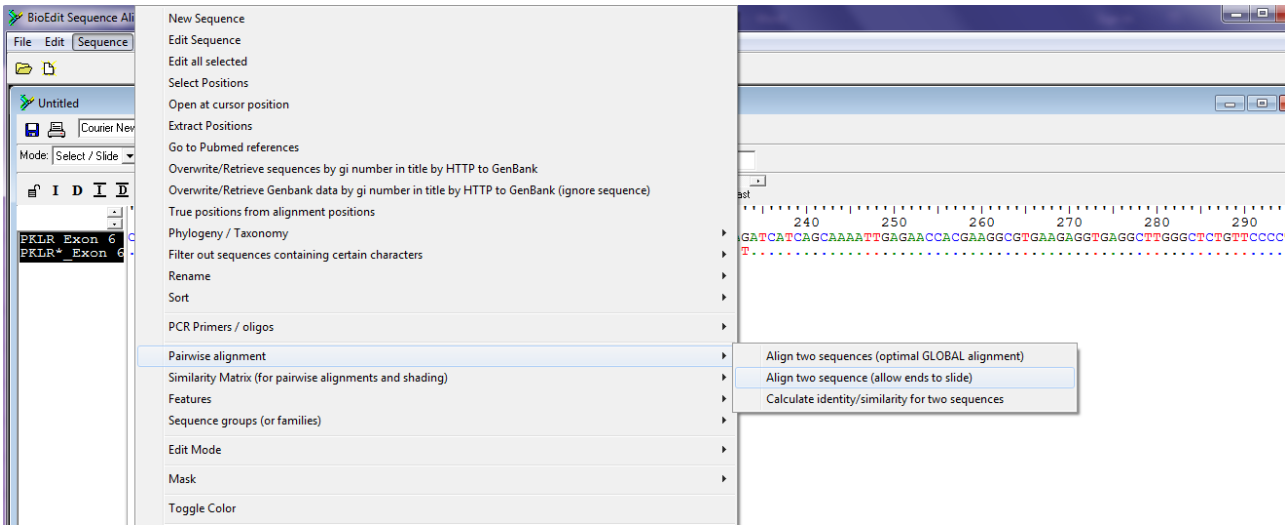


Figure 7: A mutation on exon 6- G > T at 233.

STEP 4:

The mutated Exon 6 was compared with the normal exon 6 by performing a pairwise alignment.

Click on both Exons > Sequence > Pairwise Alignment > Align two sequences (Allow ends to slide).



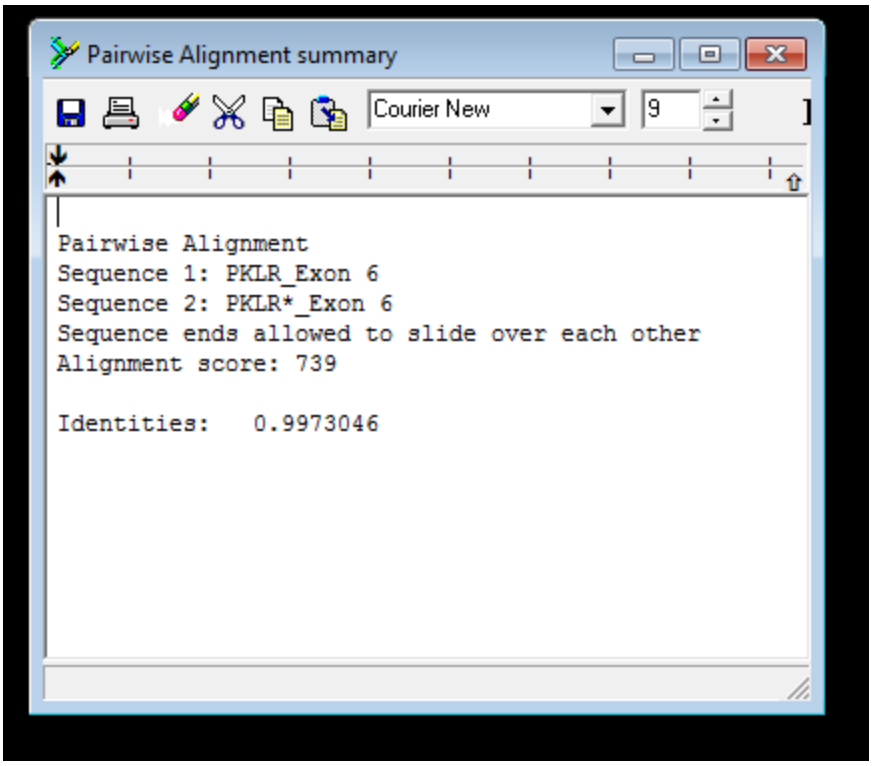


Figure 8: Pairwise alignment of the two sequences of exon 6.

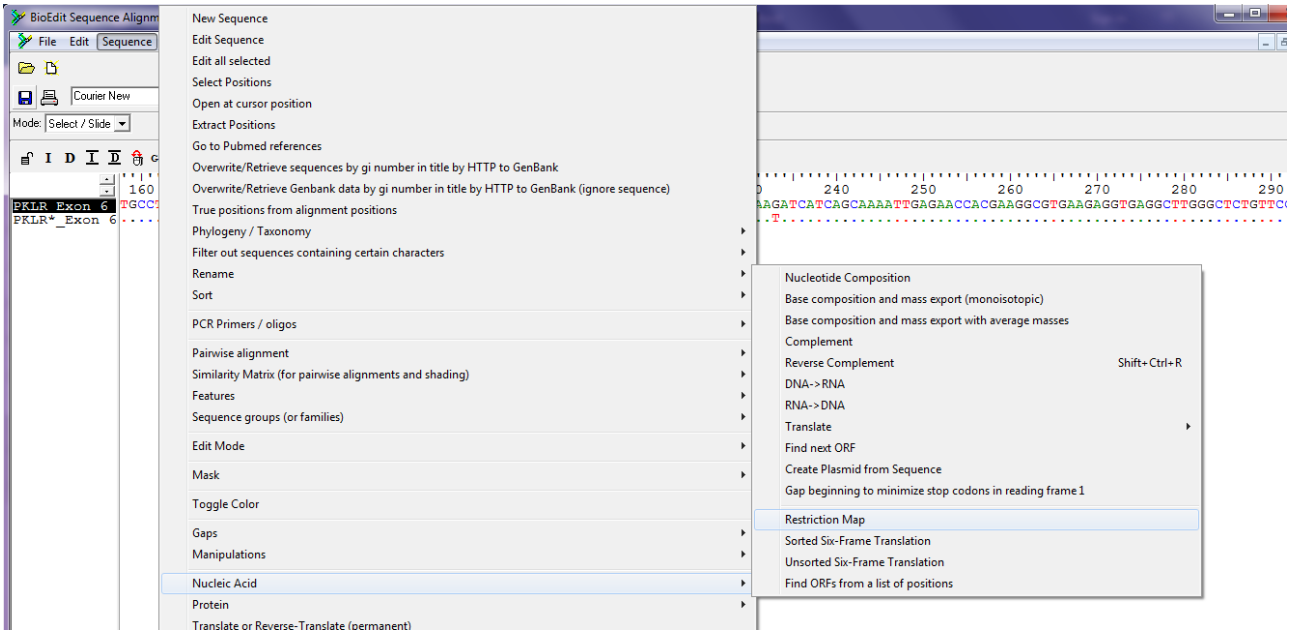
STEP 5:

Mapping of the restriction enzymes of both sequences (normal and mutated).

Click on one of the sequence to select it (for example the normal one first- PKLR_Exon 6).

Sequence > Nucleic Acid > Restriction map

On the new window that appear, Click Generate Map.



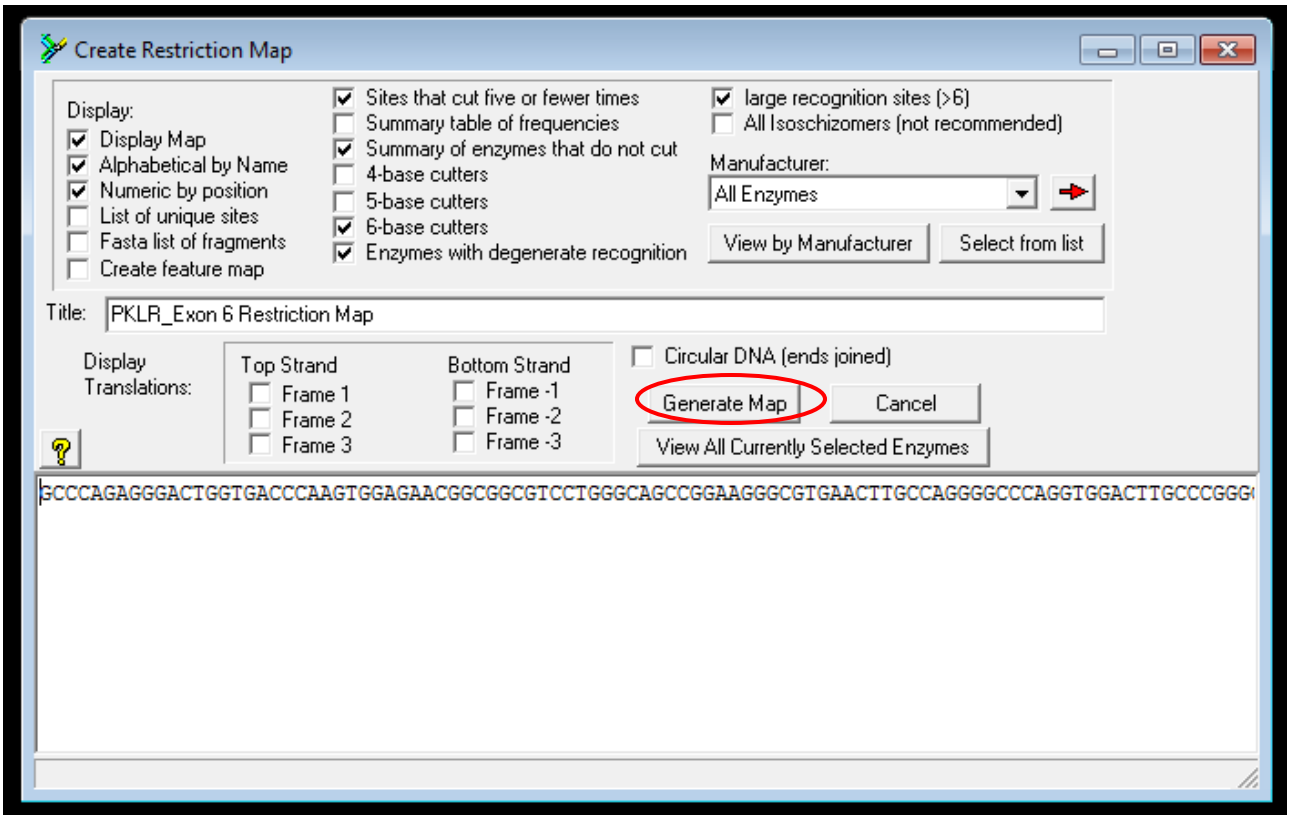


Figure 9: Getting the restriction map for normal Exon 6 (PKLR_Exon 6).

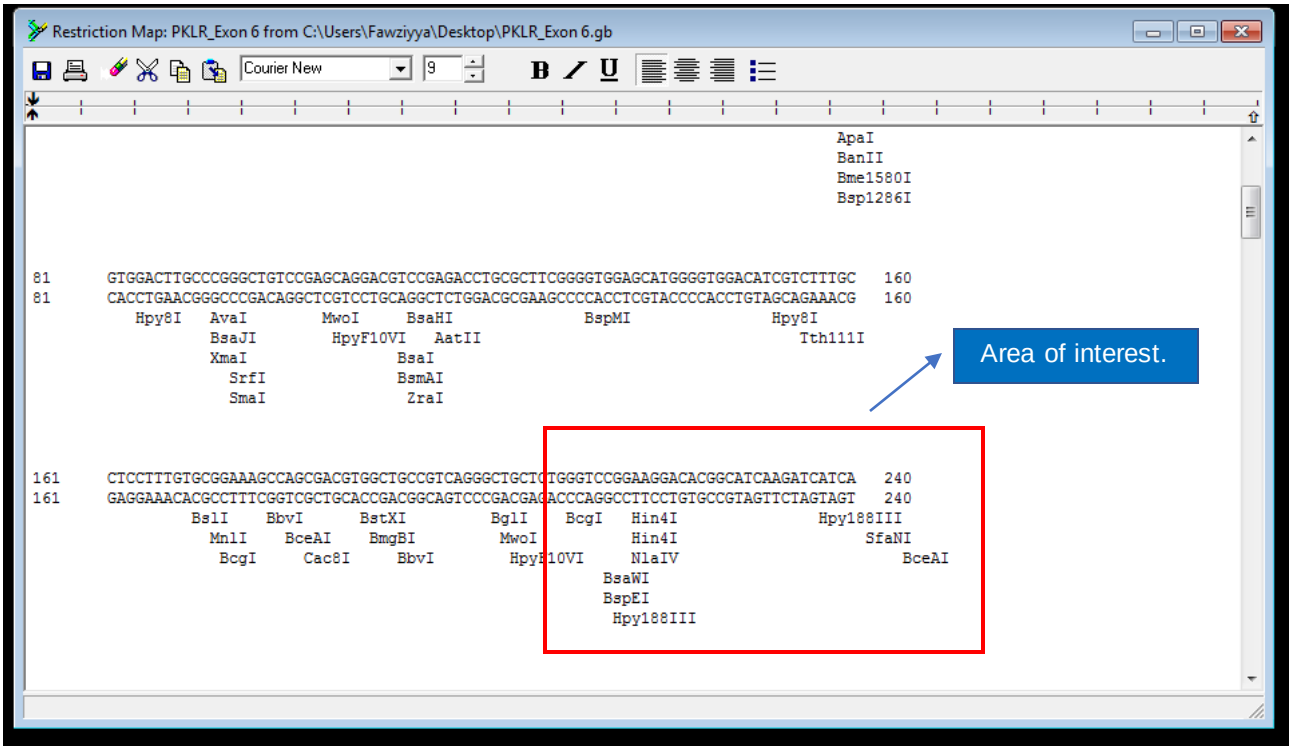


Figure 10: Restriction Map of PKLR_Exon 6.

STEP 6:

The step 5 was repeated but selecting the mutated sequence for this instance in order to get the restriction map for the mutated sequence (PKLR*_Exon 6).

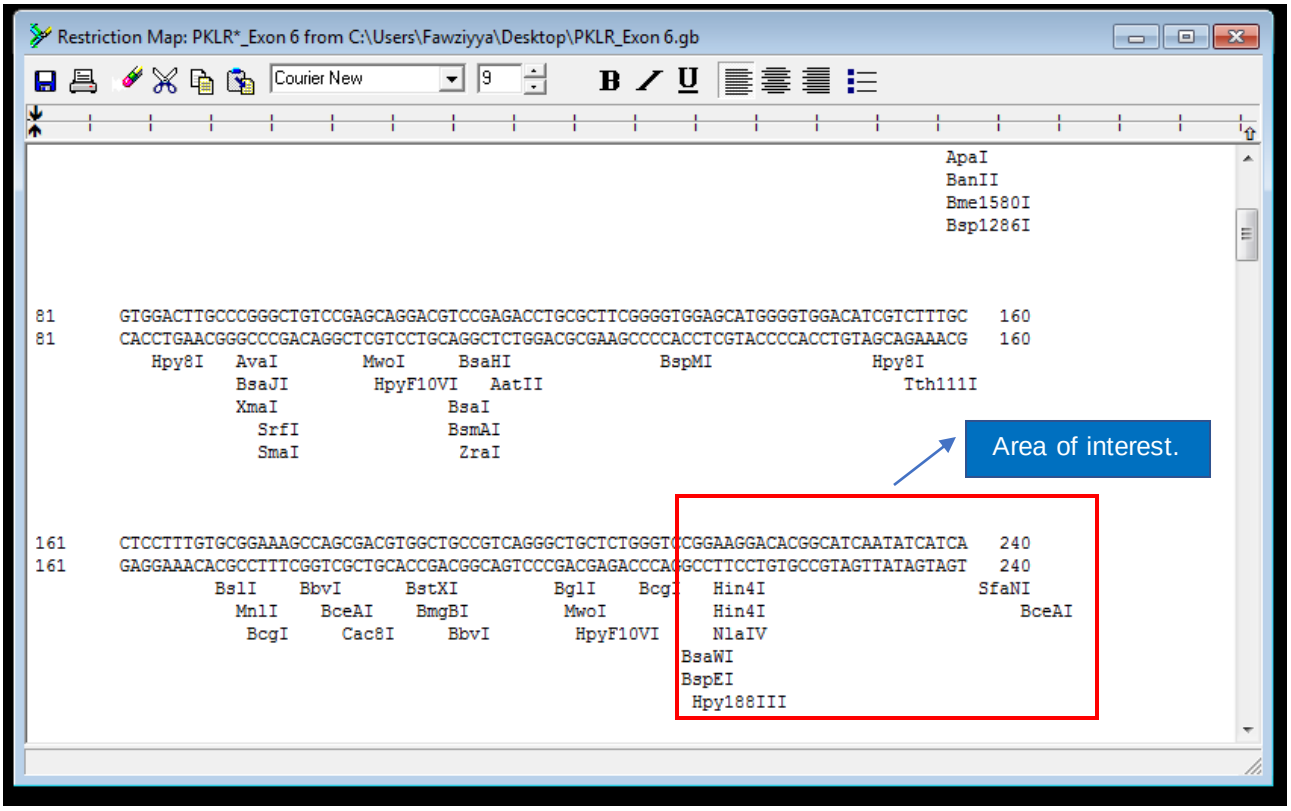


*Figure 11: Restriction Map of PKLR*_Exon 6.*

Comparison between the two-restriction map shows a change in the restriction site for the enzyme Hpy188III. To further continue the test, a restriction map was created for a second time but at this point selecting the specific enzyme Hpy188III from the list to further compare the normal sequence with the mutated sequence.

STEP 7:

Select PKLR_Exon 6

Sequence > Nucleic Acid > Restriction Map

In the new window, click on “select from list” to choose the enzyme Hpy188III

From the enzyme list, select Hpy188III and Click on double arrow (>>) to include this enzyme

Click “ok”.

Then “Generate map”

The steps are repeated for mutated sequence.

The Restriction map of both sequences were compared.

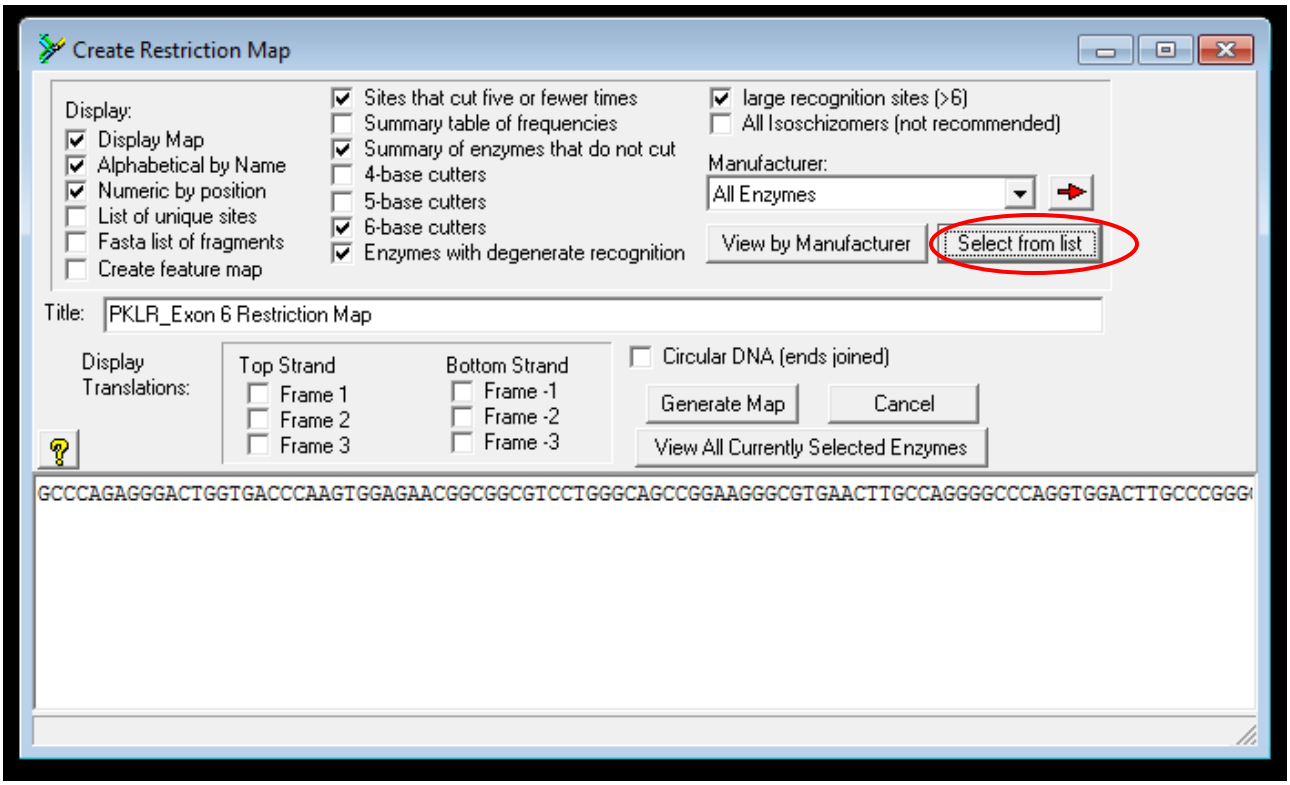


Figure 12: Restriction map with specific enzyme.

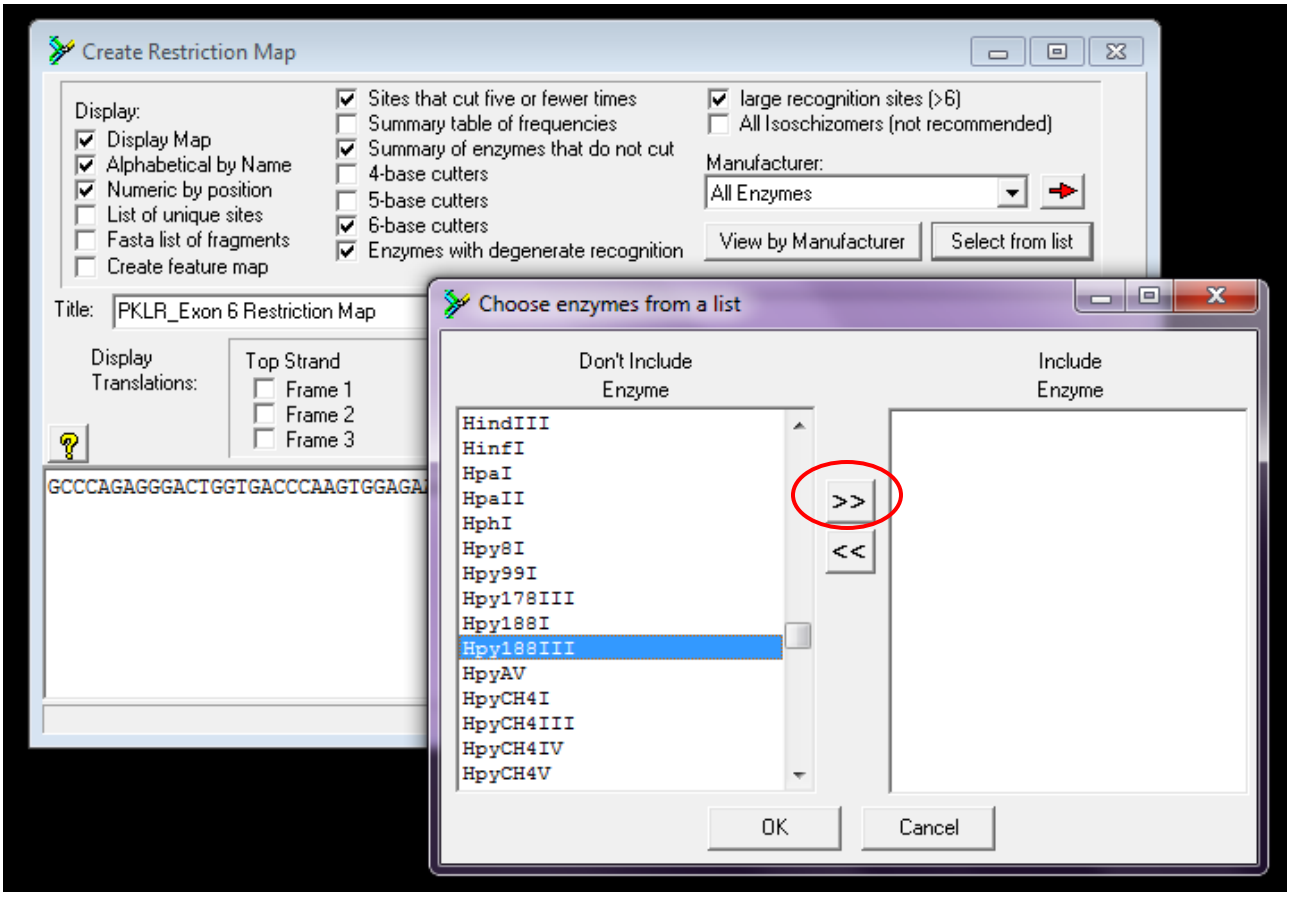


Figure 13: Selecting Hpy188III for restriction map.

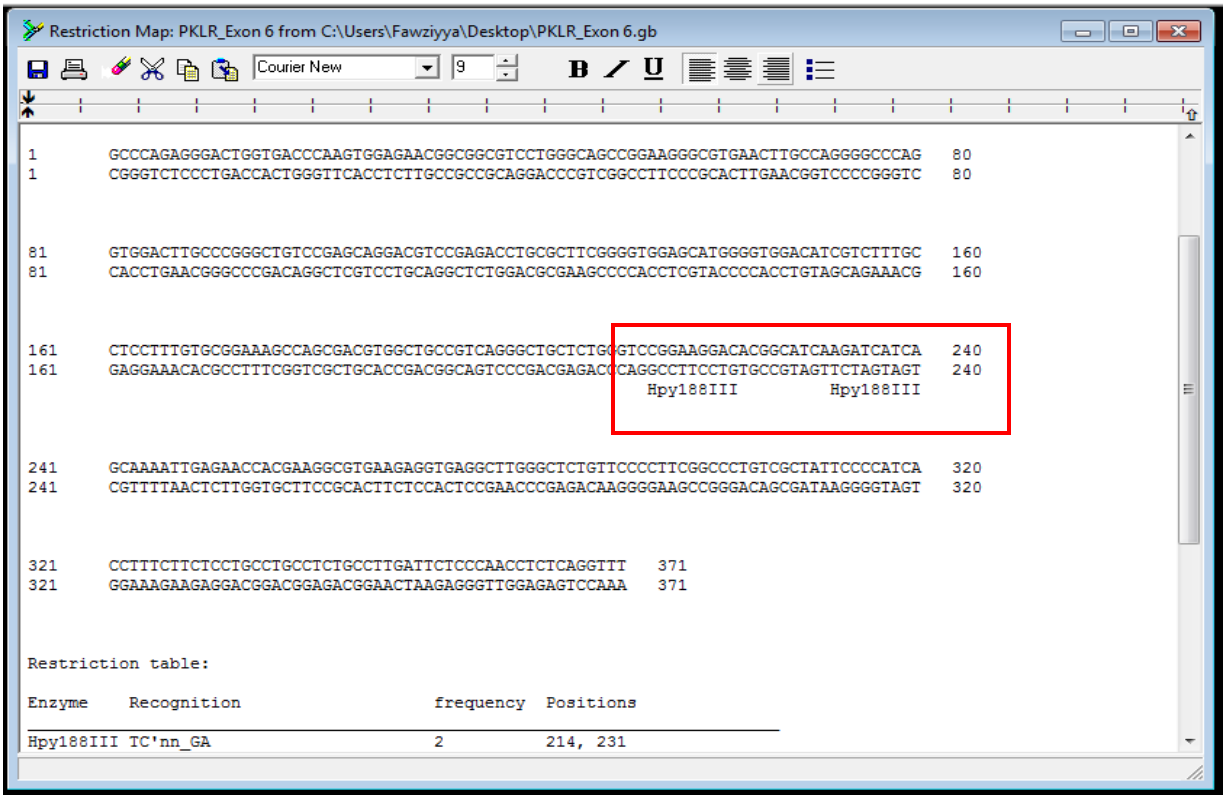


Figure 14: Restriction map for the normal Sequence- Enzyme: Hpy188III.

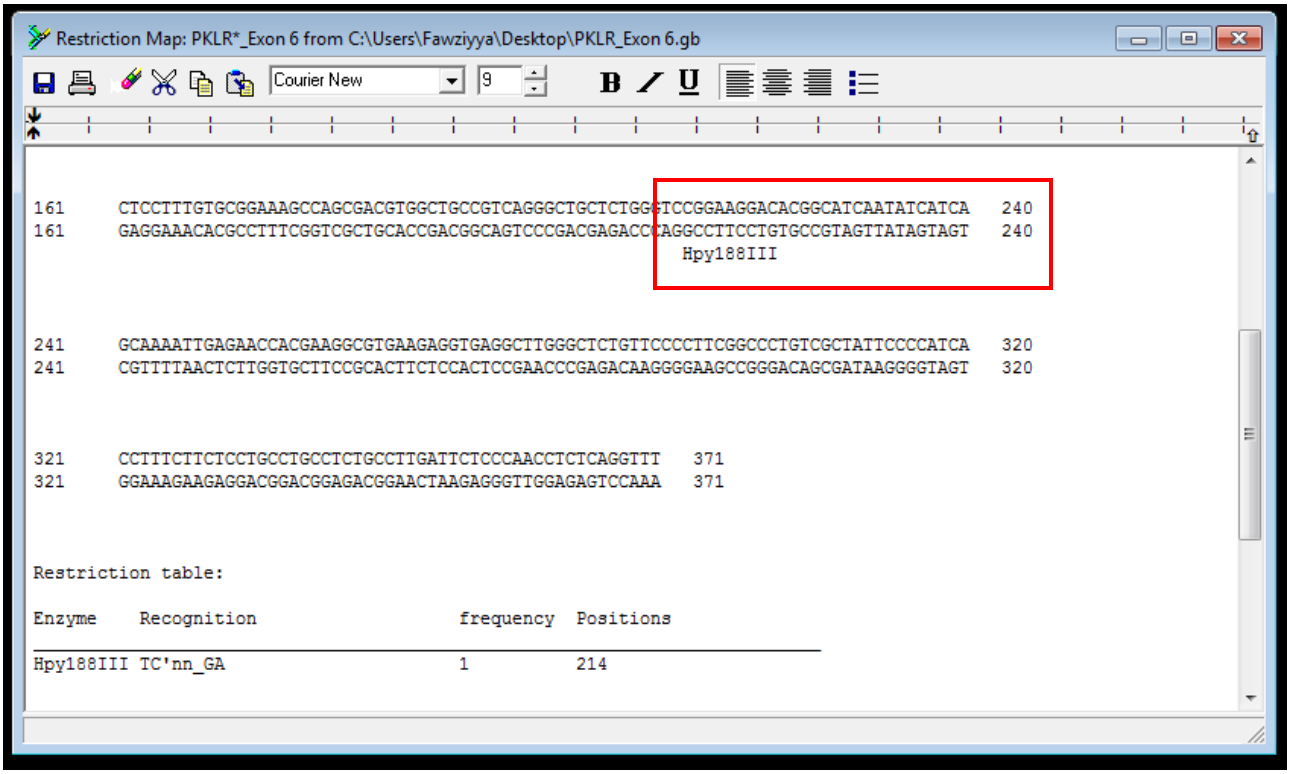


Figure 15: Restriction map for the mutated Sequence- Enzyme: Hpy188III.

Diagnostic report of PKLR Exon 6

Diagnostic report

```
Restriction Enzyme Map:

1       GCCCAGAGGGACTGGTGACCCAAGTGGAGAACGGCGGCGTCCTGGGCAGCCGGAAGGGCGTGAACTTGCCAGGGGCCCAG
80
1       CGGGTCTCCCTGACCACTGGGTTCACCTCTTGCCGCCGCAGGACCCGTCGGCCTTCCCGCACTTGAACGGTCCCCGGGTC
80


81      GTGGACTTGCCCGGGCTGTCCGAGCAGGACGTCCGAGACCTGCGCTTCGGGGTGGAGCATGGGGTGGACATCGTCTTTGC
160
81      CACCTGAACGGGCCCGACAGGCTCGTCCTGCAGGCTCTGGACGCGAAGCCCCACCTCGTACCCCACCTGTAGCAGAAACG
160
```

"G" changes to "T" (loss of restriction site for enzyme Hpy188III at 231)

```
161     CTCCTTTGTGCGGAAAGCCAGCGACGTGGCTGCCGTCAGGGCTGCTCTGGGTCCGGAAGGACACGGCATCAAGATCATCA
240
161     GAGGAAACACGCCTTTCGGTCGCTGCACCGACGGCAGTCCCGACGAGACCCAGGCCTTCCTGTGCCGTAGTTCTAGTAGT
240
                                                          Hpy188III          Hpy188III


241     GCAAAATTGAGAACCACGAAGGCGTGAAGAGGTGAGGCTTGGGCTCTGTTCCCCTTCGGCCCTGTCGCTATTCCCCATCA
320
241     CGTTTTAACTCTTGGTGCTTCCGCACTTCTCCACTCCGAACCCGAGACAAGGGGAAGCCGGGACAGCGATAAGGGGTAGT
320
321     CCTTTCTTCTCCTGCCTGCCTCTGCCTTGATTCTCCCAACCTCTCAGGTTT    371
321     GGAAAGAAGAGGACGGACGGAGACGGAACTAAGAGGGTTGGAGAGTCCAAA    371
```

Restriction table:

Enzyme	Recognition	frequency	Positions
Hpy188III	TC'nn_GA	2	214, 231

PCR product	322 bp
Normal	213 bp, 17 bp, 92 bp
Mutant	213 bp, 109 bp

Restriction enzyme

In this report, the enzyme HPy188III was considered as there is a loss of restriction site for this enzyme at position 231 in mutated sequence of PKLR exon 6. The enzyme Hpy188III recognizes the sequence "TCNNGA". Enzyme Hpy188III cuts this recognized sequence after TC (such as: TC|NNGA). This enzyme has a reaction condition requirement of 37°C. Hpy188III can be denatured by heating at 65°C for 20 minutes. *Helicobacter pylori* 188 is the genomic source for Hpy188III.

When "G" was mutated to "T" at position 233, there was a loss of restriction site. At position 233 in the mutated sequence of PKLR exon 6, the sequence changes to "TCNNTA", therefore the Hpy188III cannot recognize this mutated sequence and thus causing a loss in the restriction site at 231. Hence in the mutated sequence, only one restriction site is remained at position 214.

In the normal sequence, there are two restriction sites for the enzyme Hpy188III (at position 214 and 231). Therefore, when primer sets are used on both the normal and mutated sequence, different sizes of PCR product can be obtained.

Primer sets

The primer was obtained by using the NCBI Genome browser

Searching accession number U47654 > Get FASTA sequence

Copy FASTA sequence > Go to "Pick Primers" and Paste in "Pick primers"

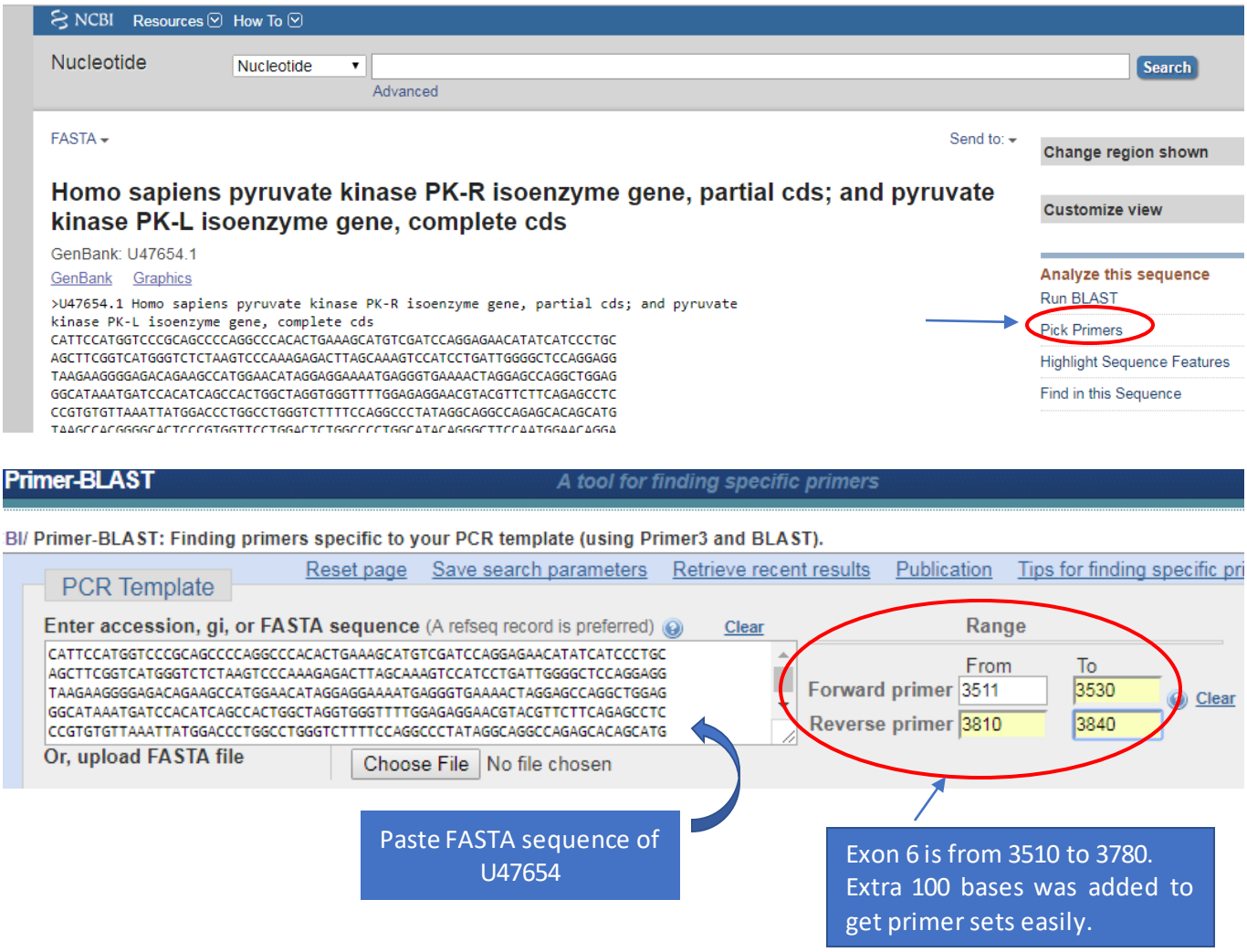
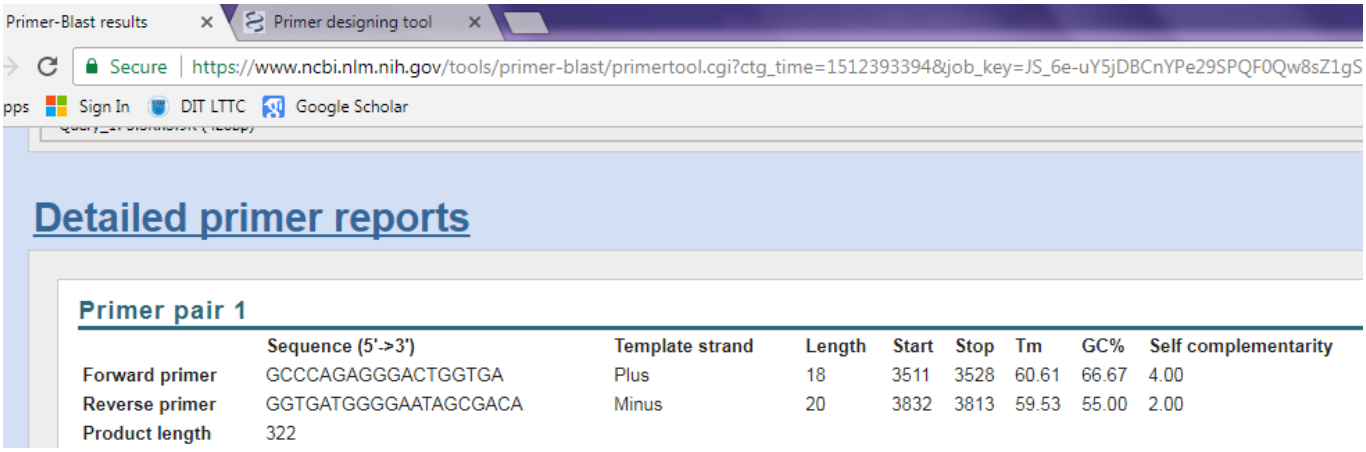


Figure 16: Getting the primer sets



	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity
Forward primer	GCCCAGAGGGACTGGTGA	Plus	18	3511	3528	60.61	66.67	4.00
Reverse primer	GGTGATGGGGAATAGCGACA	Minus	20	3832	3813	59.53	55.00	2.00
Product length	322							

Figure 17: Detailed primer reports.

The forward primer and reverse primer are highlighted in the diagnostic report section on page 25.

PCR product

PCR product is obtained from the forward primer till the reverse primer (including both primers when counting the PCR product. If there is a restriction site that the enzyme will cut, then PCR of different sizes can be obtained. In this report, the PCR product is of 322 base pairs (bp). Hyp188III cuts at TC'NNGA.

In the normal sequence, there are 2 restriction sites (at position 214 and 231) that leads to two cuts and the different sizes of PCR product: 213 bp, 17 bp, and 92 bp.

In contrast to the normal, there is only one restriction site (at position 214) in the mutated sequence of exon 6 of the PKLR gene. Therefore, in the mutated sequence, there is only one cut that produces 2 PCR product of size: 213 bp and 109 bp.

3.0 Conclusion

The various mutations on PKLR have different effect on the exons and thus causing different issues. A diagnostic test and report for the genetic mutation of exon 6 on PKLR has shown an effect on the restriction site with the use of enzyme Hpy188III. Less restriction site in the mutated sequence changes the number of splice site and simultaneously changes the number and sizes of PCR product generated compared to the normal sequence that originally had 2 restriction sites and 3 PCR products. Further diagnostic reports can be helpful by focusing on the effect of other genetic mutation on the PKLR gene.

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