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The Construction of CRISPR-Cas9 System Targeting Vector for CYP3A4 Gene in Hepatic Cell Lines

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ABSTRACT

Drug metabolism in the human liver initiated by Cytochrome P450 (CYP450) has been widely acknowledged. They oxidise drugs, harmful compounds, and endogenous molecules like steroids. In our research study, we mainly focused on CYP3A4 of the CYP450 subfamily that is widely available in the liver around 15% - 20% of hepatic content and plays a significant function in metabolising up to 50% of drugs in the market today. However, the CYP3A4 gene encodes CYP3A4 enzymes that are highly polymorphic, which could affect enzymatic activity and cause variable responses in drug metabolism. Clustered Regularly Interspaced Short Palindromic Repeats Associated Protein 9 also known as the CRISPR-Cas9 system is a gene editing method that allows scientists to edit parts of the genome by insertions and deletions. Hence, this method would be useful for genetic variants such as the CYP3A4 gene. The objectives of this study are to design a guide RNA (gRNA), to insert the designed gRNA into the pGuide-it-Zs-Green1 vector, to quantify the purified CYP3A4-KO plasmid vector by using NanoDrop® and to transfect the constructed vector in a hepatic cell line. The methodology involved the selection of the gRNA by using the online gene editing tool, *Synthego* (https://design.synthego.com), annealing oligos of the gRNA for CYP3A4, cloning gRNA into a plasmid vector, isolation, and purification of the CYP3A4-KO plasmid vector. The construction of the CRISPR-Cas9 targeting vector in this study was successfully achieved and promising since the selected gRNA for CYP3A4 gene which is 5'-ATAAATCCCACTGGACCAAA-3' and located in exon 5 was correctly ligated after the confirmed with sequencing reaction and cloned it into a plasmid vector. The yield of pCYP3A4-KO plasmid DNA was a good candidate for transfection.

1. Introduction

Cytochrome P450 (CYP) enzymes are membrane-bound haemoproteins and are known to be the heme-thiolate proteins family [1,2]. CYP450 enzymes mainly reside in the liver and circulate throughout the body. CYP450 are monooxygenases that catalyse many reactions such as drug metabolism, and synthesis of cholesterols, steroids, and lipids [3,4]. Drug metabolism is crucial as it

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converts drugs into a form that can be excreted from the body. The most prevalent cytochrome P450 (CYP) enzyme for drug metabolism in the liver and small intestine is CYP3A4 (cytochrome P450, family 3, subfamily A, polypeptide 4) [3,5]. Additionally, it contributes to the metabolism of 45–60 commonly used therapeutic medicines [5]. About half of all currently prescribed medications, including acetaminophen, codeine, cyclosporin A, diazepam, erythromycin, and chloroquine, are metabolized by the CYP3A4 enzyme [3]. CYP3A4 should be given special attention, especially in many pathological conditions like lung cancer and chemotherapy [6,7].

CYP3A4 are enzymes that are encoded by the CYP3A4 gene initially located in chromosome 7q22.1 [3,5]. This enzyme is frequently expressed in the liver and small intestine, where it is expressed differently in the livers of different people more than 40 times [5]. The metabolism of CYP3A4 substrates varies by up to 10 times between different people in vivo, indicating that the enzymes are highly polymorphic [5,8]. CYP3A4 gene undergoes polymorphism due to 856 SNPs (single nucleotide polymorphism) in an analysis that involved 141,456 individuals [9].

Polymorphism of the CYP3A4 gene can be unpredictable especially in the statins family because they primarily undergo the CYP3A4 metabolic pathway [10]. CYP3A4 gene polymorphisms specifically in CYP3A4*22 may be linked to decreased CYP3A4 enzymatic activity and further lipid reduction in simvastatin users [11]. The bioavailability of simvastatin lactone prodrug may be enhanced by a single nucleotide polymorphism in CYP3A4*22 that leads to a rise in plasma concentrations of simvastatin lactone and simvastatin acid [12]. Furthermore, there is a link between CYP3A4 genetic polymorphism and sufentanil consumption, which could help optimize anaesthesia strategies and reduce side effects [13]. The study concluded that individuals with CYP3A4*1G/*1G had lower sufentanil plasma concentration than CYP3A4*1/*1 and CYP3A4*1/*1G due to reduction of CYP3A enzymatic activity [13]. The polymorphism of CYP3A4 such as CYP3A4*1G/*1G genotype could be used as a marker to develop a personalised anaesthesia strategy, which could improve anaesthesia efficiency while decreasing anaesthetic side effects [13].

It is well known that CYP3A4 are highly polymorphic and could variably affect the substrates of CYP3A4 enzymes to give better or worse outcomes of drug response. However, to a certain extent, we could knockout (KO) the expression CYP3A4 gene to determine other roles of the gene that would give certain insights into drug metabolism by constructing a CRISPR-Cas9 system targeting vector for CYP3A4. Gene KO is a technique that permanently prevents the expression of a specific gene by altering the genomic DNA of a cell or model organism. Unlike knockdown methods, gene knockout methods damage specific genes, rendering them inactive. If the cells or model organisms survive the knockout, the cells unable to express the functional gene product again. As a result, unwanted polymorphism and mutation would be reduced.

CRISPR-associated 9 (Cas-9) enzyme previously discovered in *Streptococcus pyogenes* as part of its adaptive immunity system is used to cleave target DNA, resulting in a double-strand break (DSB) [14-16]. The Cas-9 enzyme is directed by the guide RNA (gRNA), which consists of a 20-nucleotide sequence (the protospacer) that is complementary to the genomic target sequence [15,16]. A 3' protospacer-associated motif (PAM), e.g.: NGG is found next to the genomic target sequence and is required for Cas9 binding and cutting [15,16]. Cas9 cleaves both strands of DNA after binding to the DNA target, activating repair mechanisms that can be used to modify the locus of interest. Cells attempt to repair DSBs to avoid cell death caused by loss of genomic stability. However, repair of the DSB does not always imply complete repair. Gene KO via CRISPR genome editing would not work if perfect repair occurred every time. Instead, when DSBs are repaired, insertions and deletions (INDELS) that introduce stop codons and gene knockouts typically occur [16].

In 2018, CRISPR-Cas9 has recently been used in investigations of drug absorption, distribution, metabolism, and excretion (ADME) as well as the creation of ADME models. From the same study,

twenty of the studies discuss gene editing of clinically important genes including ATP-binding cassette drug transporters and cytochrome P450 drug-metabolizing enzymes. The ADME toolbox has been significantly enlarged due to CRISPR-Cas9 and this new method allows us to create more accurate and predictive ADME models in vitro and in vivo, as well as map that leads to unknown ADME genes and gene families [17]. A 2016 study produced CYP2E1 KO in rats using the CRISPR-Cas9 system indicated no expression and loss of function in the CYP2E1 enzyme [18]. From this outcome, the metabolism of CYP2E1 substrates was inactive and the KO CYP2E1 rats might give more insights into further chemical metabolism, toxicity, carcinogenicity and its roles in drug-drug interaction [18]. A study in 2017 performed a CYP3A1/2 KO in rats using the CRISPR-Cas9 method resulted in the absence of CYP3A1/2 functionality when compared with the wild-type (WT) rats [19]. This study could benefit future researchers to study the physiology and pharmacology of CYP3A drug metabolism.

There was an in vitro model in a human hepatocyte-derived cancer cell line that had been effectively created to utilize CRISPR-Cas9 by expressing the CYP3A5 with the conversion of CYP3A5*5 to activate CYP3A5*1 instead of KO. The successful gene editing of CYP3A5 resulted in elevated CYP3A5 mRNA levels and increased metabolism of two CYP3A5 substrates; midazolam and tacrolimus [20].

Recent reports in 2020 showed that the CRISPR-Cas9 technique was utilized to remove the orthologous genes of CYP2J2 in humans, and rat CYP2J3/10 [21]. In the same study, the CYP2J3/10 knockout rats were alive and productive, and no off-target effects were seen. The mRNA and protein expression of CYP2J3/10 in the liver, small intestine, and heart of knockout rats were completely absent as compared to WT rats [21]. CYP2J4 mRNA and protein expression were both considerably reduced in these tissues at the same time. Further in vitro and in vivo metabolic tests of astemizole, a common CYP2J substrate, revealed that in knockout rats, CYP2J was functionally inactive [21]. In 2022, geranylgeranoic acid (GGA) responsible for inhibitors against second primary hepatoma were unexpectedly maintained after the KO of monoamine oxidase B (MAOB) cells by CRISPR-Cas9 [22]. After being investigated, the authors concluded that CYP3A4 expression was increased after MAOB-KO and CYP3A4 were found to act as alternative oxidases to geranylgeraniol subsequently maintaining the GGA. Thus, the study of KO cells might result in alternative functions of the enzymes to adapt to the metabolism of substrates or drugs in the body.

However up till today, there is no *in vitro* study on CYP3A4 gene KO using CRISPR-Cas9 has been reported. Our study is still significant since the data of CYP3A4 gene KO in cell lines are still lacking. Thus, in this study we aim to design the desired gRNA of the CYP3A4 gene, to insert the gRNA of CYP3A4 into delivering vector pGuide-it-ZsGreen1 (linear) vector by Takara Bio to become CYP3A4-KO plasmid vector, to quantify and to transfect CYP3A4-KO plasmid vector in a hepatic cell line.

This new gene-editing tool such as CRISPR is a brilliant, affordable, and simple tool that can be used in future KO CYP450 sub-gene. For example, the CYP3A4 gene will provide more inputs on drug metabolism after KO. This study was carried out to observe CRISPR-Cas9 simplify the functional pathways of the CYP3A4 gene.

2. Methodology

2.1 Materials

Two oligos of CYP3A4 for the KO target site (25nmole DNA, 24 bp for each) 5'-ATAAATCCCACTGGACCAAA-3' and 5'-TTTGGTCCAGTGGGATTTAT-3' respectively were obtained from Integrated DNA Technologies, Inc. Guide-it CRISPR-Cas9 System (Green) (Cat. No. 632601) kit from Takara Bio Company consists of pGuide-it-ZsGreen1 vector (Linear) (7.5 ng/µl), Guide-it Ligation Components such as DNA Ligation Mighty Mix, Guide-it Oligo Annealing Buffer, Guide-it Control

Annealed Oligos (100 fmol/µl), Guide-it Sequencing Primer 1 (100 pmol/µl), PCR-Grade Water. The kit also contains Stellar Competent Cells (100 µl/tube) and SOC Medium (1ml/tube). For DNA purification, Monarch® Plasmid Miniprep Kit – NEB and Bio Basic Plasmid DNA Extraction Maxiprep Kit were used.

2.2 Target Selection for Guide RNA (gRNA)

The Cas9 nuclease's specificity was determined by the gRNA's 20-nucleotide guide sequence. An appropriate knockout guide design tool (https://design.synthego.com) was used to build an appropriate target sequence. Three top-ranked guide RNAs were recommended to target the CYP3A4 gene in the Homo sapiens genome as in Table 1.

Table 1
Recommended guide RNAs for knockout in CYO3A4

Top-ranked gRNA (5' – 3')
ATAAATCCCACTGGACCAAA
ACTCTAGCCTTTTGGTCCAG
CTCTAGCCTTTTGGTCCAGT

To boost the likelihood of successful genome editing, more than one gRNA has been suggested. The gRNAs recommended were all found on exon 5 as shown in Figure 1.

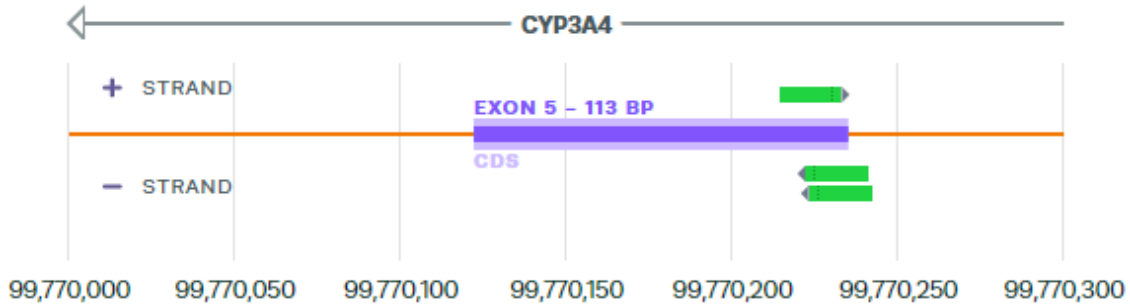

Fig. 1. Recommended location of gRNAs for knock out of CYP3A4 gene at Exon

2.3 Plasmid Construction

2.3.1 Annealing of gRNA

Each gRNA sequence was thoroughly resuspended in TE buffer or molecular-biology grade, nuclease-free water at a concentration of 100 µM. The gRNAs were then combined in a 200 µl PCR tube containing 1µl gRNA 1 (100 M), 1 µl gRNA 2 (100 M), and 8 µl Guide-it gRNA Annealing Buffer, for a total volume of 10 µl. Following that, gRNAs were annealed using a thermal cycler to denature at 95°C and then reannealed by gradually lowering the temperature. The thermal cycler has been configured with the following cycling conditions: 95°C for 2 minutes, a 10-minute slope from 85°C to 30°C, and a hold when it reaches 25°C. Following that, 1 µl of the annealed gRNA solution was mixed with 99 µl of Guide-it gRNA Annealing Buffer to create a 100 nM (fmol/l) solution. The annealed gRNAs were kept at –20°C.

2.3.2 Ligation of gRNA into delivering vector

The necessary reagents were thawed at room temperature, and the reaction was set up to a total volume of 10 µl, which includes 2 µl pGuide-it vector (Linear) (7.5 ng/µl), 1 µl Target annealed gRNAs (100 fmol/µl) or Guide-it Control Annealed Oligos (100 fmol/µl), 2 µl PCR Grade Water and 5 µl DNA Ligation Mighty Mix. The reaction mixture was incubated for 30 minutes at 16°C. In the meantime, one vial of Stellar Competent Cells had been defrosted on ice.

2.3.3 Bacterial transformation process

The entire 10 µl ligation mixture was poured into the competent cells, gently mixed by tapping, and left on ice for 30 minutes. The cells had been shocked at 42°C for 45 seconds before being placed on ice. A 2-minute incubation period had been done.

After that, 1 ml of SOC medium was added, and the mixtures were incubated at 37°C for 1 hour with vigorous shaking. Then, 100 µl of the culture was plated on pre-warmed (37°C) LB plates with ampicillin (final concentration 100 µg/ml). The plates had been incubated overnight at 37°C.

2.3.4 Isolation and purification of plasmids

Two single colonies were selected and inoculated into two separated 7 ml of LB broth medium containing ampicillin (final concentration 100 µg/ml) and were labelled as A1 and A2 for CYP3A4. After that, it had been shaken overnight at 37°C to grow the bacterial cells at 200 rpm. Purification of plasmid DNA had been performed using Monarch® Plasmid Miniprep Kit – NEB according to the kit's protocol. Next, we quantified plasmid DNA to determine its purity and concentration through 260/280 absorbance measures using a NanoDrop Lite Spectrophotometer (Thermo Scientific). For validation, we sequenced plasmid DNA containing gRNA through sequencing reaction using primer 5'-GAGGGCCTATTTCCCATGATTCC-3' that primes at U6 promoter downstream the gRNA insert. Lastly, we maximised the DNA plasmid to yield larger quantities using Bio Basic Plasmid DNA Extraction Maxiprep Kit and determined its purity and concentration for further transfection process.

3. Results

3.1 Selected gRNA Based on Synthego Result

In this section, we discussed the selected gRNA. The gRNA with the highest score, according to *Synthego* was chosen for oligo 1 and its complementary was chosen as oligo 2 as shown in Table 2).

Table 2
The selected gRNA for CYO3A4

CYP3A4 Guide RNA (5' – 3')	
Oligo 1	ATAAATCCCACTGGACCAAA
Oligo 2	TTTGGTCCAGTGGGATTTAT

3.2 Annealing of the Oligos

In this part, gRNAs were assembled by annealing the oligo 1 and oligo 2 and ligated them to complementary restriction site overhangs such as 5' overhangs sequence, ccgg and the antisense 5' overhang sequence, aaac of the plasmid pGuide-it-ZsGreen1 vector as illustrated in Figure 2.

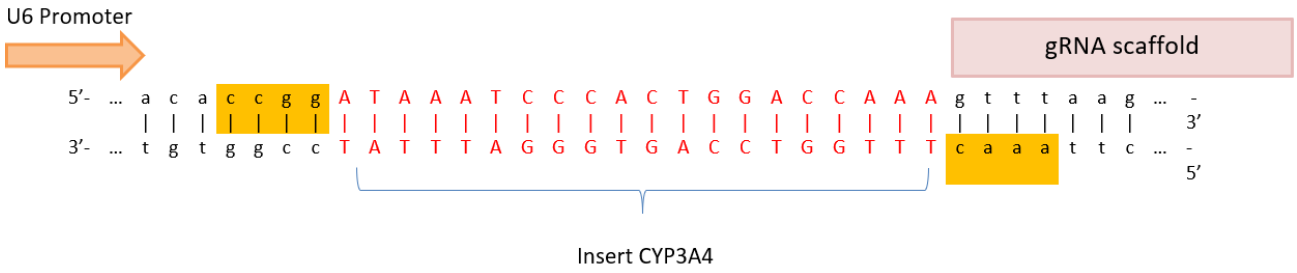

Fig. 2. Ligation gRNA of CYP3A4 to 5' overhangs sequence, ccgg and the antisense 5' overhang sequence, aaac of the plasmid pGuide-it-ZsGreen1 vector

3.3 Ligation and Bacterial Transformation

Oligo 1 and oligo 2 of CYP3A4 that had been successfully annealed or can be called target insert of CYP3A4 that was prepared before cloning into pGuide-it-ZsGreen1 vector through ligation and bacterial transformation. After transformation, there was a growth of colonies after incubation on LB plates with ampicillin at a final concentration of 100 µg/ml as shown in Figure 3.

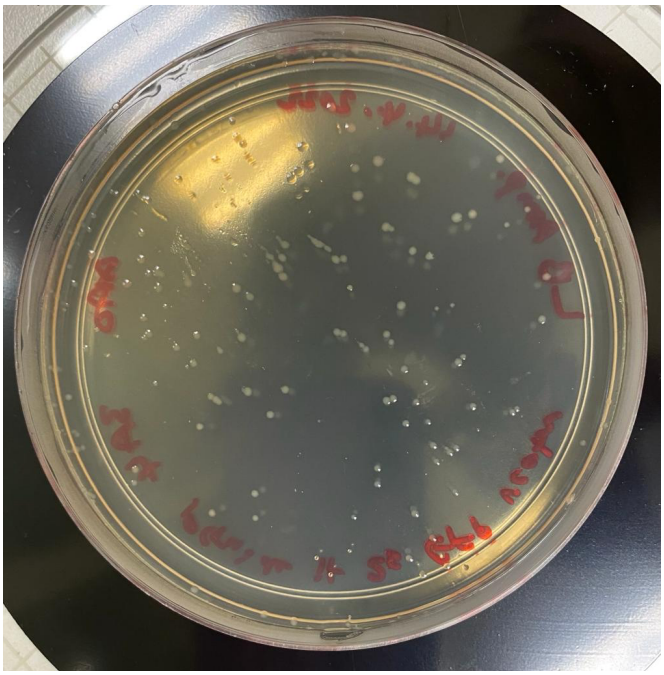

Fig. 3. Successful bacterial transformation with the growth of colonies

3.4 Quantitative Analysis of Purified CYP3A4-KO

Quantitative analysis of purified CYP3A4-KO plasmid vector sample A1 and sample A2 after bacterial transformation that had been isolated is in Table 3.

Table 3
Quantification of purified CYP3A4-KO plasmid vectors based on NanoDrop® Lite Spectrophotometer at 260 nm absorbance

	Sample A1		Sample A2	
	Concentration (ng/µl)	A260/280	Concentration (ng/µl)	A260/280
Mini-prep	135.5	1.85	215.1	1.34
Maxi-prep	2787.2	1.35	2753.3	1.70

To collect tiny plasmid DNA from bacteria while reducing contaminating proteins and their genomic DNA, the mini-prep technique was used before proceeding with the sequencing reaction. The cloning of target inserts into the pGuide-it-ZsGreen1 vector for both sample A1 and sample A2 had been a success after had been confirmed by sequencing reaction. Evidence of pGuide-it- ZsGreen 1 vector with target insert of CYP3A4 had been successfully ligated as illustrated in Figure 4.

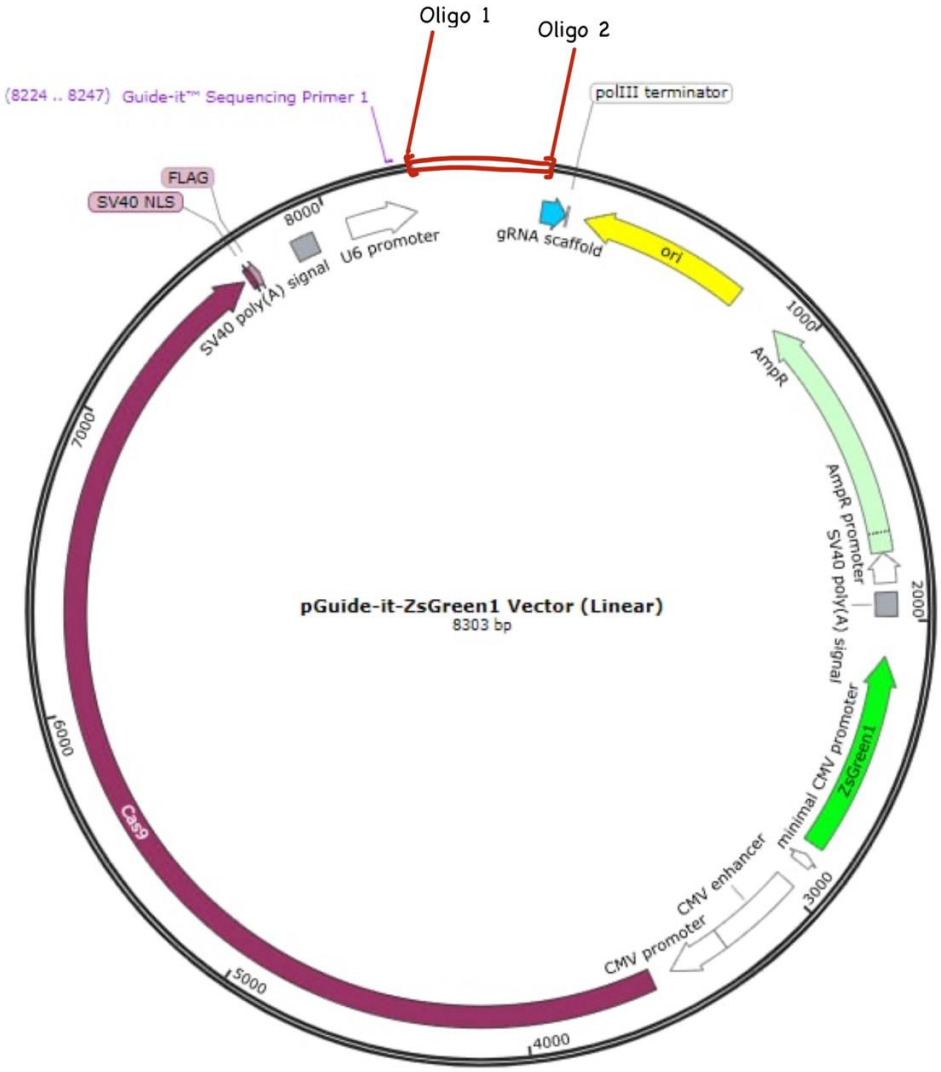

Fig. 1. pGuide-it-ZsGreen1 vector with target insert of sample A1 CYP3A4 after sequencing

3.5 Sequencing Reaction of CYP3A4-KO

The Chromatogram of the CYP3A4-KO plasmid vector with the highlighted sequence indicated the target insert of CYP3A4 as shown in Figure 5. The reference sequence of the pGuide-it-ZsGreen1 vector with the target insert had been designed for control purposes through the *in-silico* method.

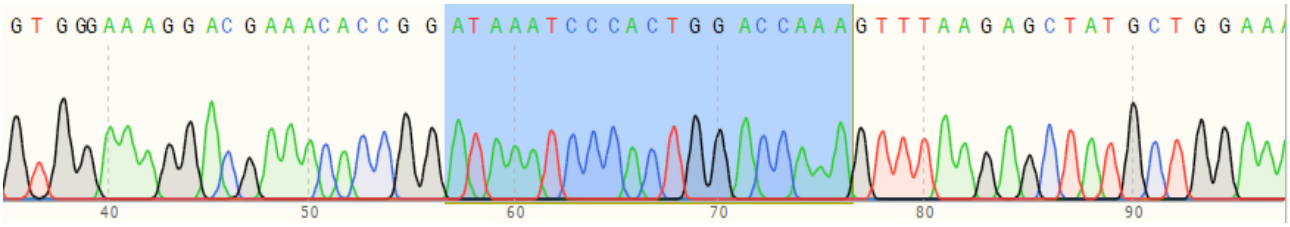

Fig. 5. Chromatogram of CYP3A4-KO plasmid vector with highlighted target insert of CYP3A4

The alignment of the reference sequence with sample A1 and sample A2 CYP3A4-KO plasmid vectors has been demonstrated in Figure 6. We observed that the gRNA or target insert for CYP3A4 highlighted was well-aligned with the reference sequence. We also observed there was a point mutation of insertion in the U6 promoter region for both samples as shown in the red box.

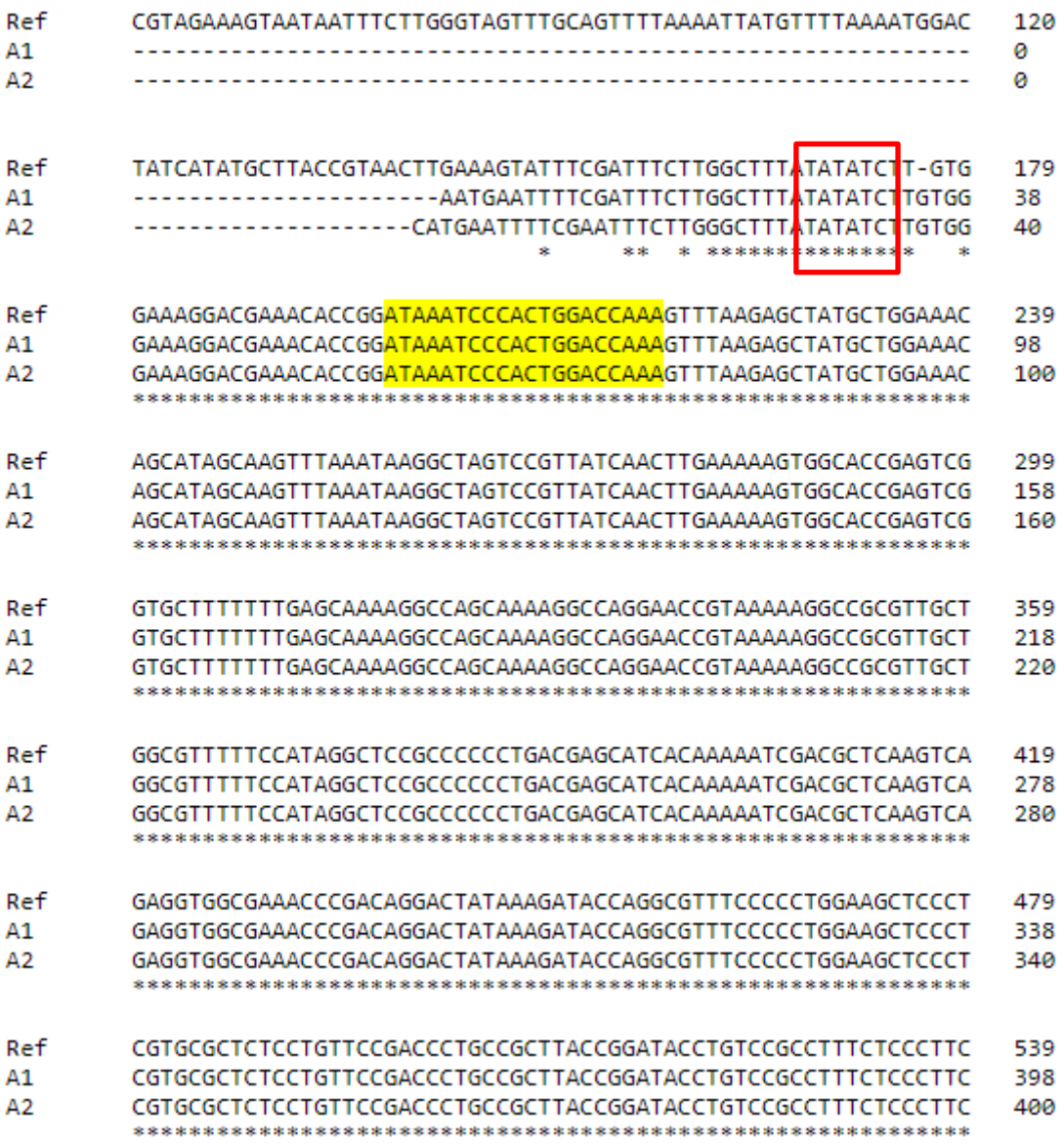

Fig. 6. Alignment of reference sequence with sample A1 and sample A2 of CYP3A4-KO plasmid vectors

4. Conclusions

In summary, we have successfully designed gRNA of CYP3A4 for the KO target site by using *Synthego* (https://design.synthego.com), 5'-ATAAATCCCACTGGACCAAA-3' at Exon 5 with an on-target score of 0.475. The sequencing result also demonstrated that our gRNA target insert had been successfully incorporated into the pGuide-ZsGreen1 Vector. Purification of plasmid from bacterial cells by using mini-prep and maxi prep had been encouraging since the concentration and purity of CYP3A4-KO plasmid vectors had been quantified using NanoDrop Lite Spectrophotometer. Thus, CYP3A4-KO plasmid vectors that had been purified and quantified could be used in the next step of transfection in future research. Thus, we expected that using the CRISPR-Cas9 system in CYP3A4 gene KO will greatly simplify the functional analysis of genes, pathways, and families, especially for the CYP3A4 gene in future drug metabolism research with genetic variants.

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