Tetra-primer amplification refractory mutation system (ARMS) PCR used to detect 3'UTR rs1948 mutation in *CHRNB4*

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ABSTRACT Rs1948 A>G is a single nucleotide variation (SNV) in the 3'-UTR of *CHRNB4*. Genotyping the synonymous *CHRNB4* rs1948 may be useful in identifying a lung cancer susceptibility gene. The study aimed to develop a simple and easy tetra-primer amplification refractory mutation system (ARMS PCR) for *CHRNB4* rs1948. The following steps were taken to optimize tetra-primer ARMS PCR: 1) determining the gene sequence and position of a single mutation; 2) developing outer and inner primers; 3) amplification of target gene fragments via PCR using an outer primer; 4) genotyping PCR product using Sanger sequencing; 5) determining the optimal annealing temperature and PCR cycle; 6) determining optimal outer and inner primer ratio; and 7) testing the reproducibility of the PCR program and final validation with Sanger sequencing. Genotype (PCR result) was visualized with 3% agarose gel electrophoresis. Optimum condition was determined as annealing temperature of 64.8 °C and 35 cycles, outer and inner primer ratio of 1:6, and DNA volume of 3 µL. Sanger sequencing confirmed the results of the tetra-primer ARMS PCR and it was shown that ARMS PCR was able to identify three different variants of *CHRNB4* rs1948.

KEYWORDS *CHRNB4*; genetic variant; genotyping; nicotinic receptor; tobacco smoking; rs1948

1. Introduction

It is known that genetic factors strongly influence tobacco addiction, by acting through nicotinic acetylcholine receptors (nAChR) (Gallego et al. 2013). Several studies showed that genetic variants in nAChR subunit genes (*CHRN* genes) were linked to the development of lung cancer (Berrettini and Doyle 2012; Jensen et al. 2015; Schaal and Chellappan 2016). Previous studies have widely reported an association between genetic variation at the 15q25.1 locus containing the *CHRNA3-CHRNA5-CHRNB4* gene cluster with nicotine dependence, alcohol drinking, and smoking-related illnesses (Broms et al. 2012; Kapoor et al. 2012; Lee et al. 2018). In particular, the *CHRNB4* has been reported to be strongly associated with the genetic lung cancer susceptibility gene (Scherf et al. 2013). One of the most notable reported variants in this gene was rs1948 (Broms et al. 2012; Lee et al. 2018; Sun et al. 2018).

CHRNB4 rs1948 is a single nucleotide variation (SNV) that changes adenine (A) allele to guanine (G) allele. This mutation is located in the 3'-untranslated regions (UTR) of *CHRNB4*. Sun et al. (2018) stated that this vari-

ant may affect the stability of mRNA due to its location. The heterozygous CT genotype was found to increase the risk of lung cancer in the non-smoker China Han population (adjusted OR = 1.896, 95%CI = 1.069-3.362).

Identifying different genotypes of *CHRNB4* rs1948 variant can be done with different methods, including conventional amplification refractory mutation systems (ARMS) polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), qPCR, or sequencing. Sequencing method is the most precise technique to detect mutations, but it is costly when there are many samples. The conventional ARMS PCR technique is less expensive, but it has low accuracy. The RFLP method uses conventional PCR with genotyping determined by the use of restriction enzyme, but it has limits in reproducibility, enzyme availability and cost. In recent years, the combination of the principle of tetra-primer PCR and the ARMS techniques has been made to identify mutations. Named tetra-primer ARMS PCR, this technique uses four primers, two outer primers and two inner primers, which are run like conventional PCR followed by gel agarose electrophoresis. This technique has good accuracy and is efficient in detecting mutations when optimized

and has been validated (Honardoost et al. 2014). This article describes a novel strategy in optimizing the tetra-primer ARMS PCR to detect 3'UTR mutation of *CHRNB4* rs1948.

2. Materials and Methods

The experiment was conducted under the approval of the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada with the number of KE/0922/EC/2019.

2.1. DNA *isolation*

A total of 13 DNA samples were isolated from the peripheral blood of volunteers. Three samples (C1, C2 and C3) were used in the tetra-primer ARMS PCR optimization step, while 10 samples (S1-S10) were used in the reproducibility step. QIAmp DNA Mini Kit (50) No. 51304 (Qiagen, United States) was used to isolate the DNA. The procedures followed the instructions of the kit. DNA concentration was checked using a spectrophotometer: NanoDrop (Thermo Fisher Scientific, USA). DNA quality and intactness were checked in two percent gel agarose electrophoresis (Agarose: GeneDireX, USA).

2.2. Primer design

Detailed information of the *CHRNB4* rs1948 variant can be found in Table 1. The minor allele frequency (MAF) of rs1948 was checked in several genome projects related to the Indonesian population. This step was taken to ensure that the variant was present in our population pool. DNA sequence of *CHRNB4* rs1948 was searched on the NCBI database (reference sequence: NC_000015.10; https://www.ncbi.nlm.nih.gov/nuccore/NC_000015.10?from=78623282&to=78655586&report=genbank&strand=true) and used as reference for primer design (Figure 1). Two distinct sets of outer and inner PCR primers (later referred to as "primers") were designed by using the web-based software "Primer1" (http://primer1.soton.ac.uk/publichtml/primer1.html) according to Ye et al. (2001). Outer primers' specificity to detect the *CHRNB4* gene was tested using NCBI BLAST program (http://www.ncbi.nlm.nih.gov/blast). A schematic illustration of the Tetra-primer ARMS PCR for *CHRNB4* rs1948 and the expected size of PCR product can be seen in Figure 1.

TABLE 1 CHRNB4 rs1948 variant information.

Gene Variant	Position	Region	Changes	Allele
CHRNB4 rs1948	chr15:78625057	3' UTR	c.594 T>C	A/G

*All information was taken from NCBI database (http://www.ncbi.nlm.nih.gov).
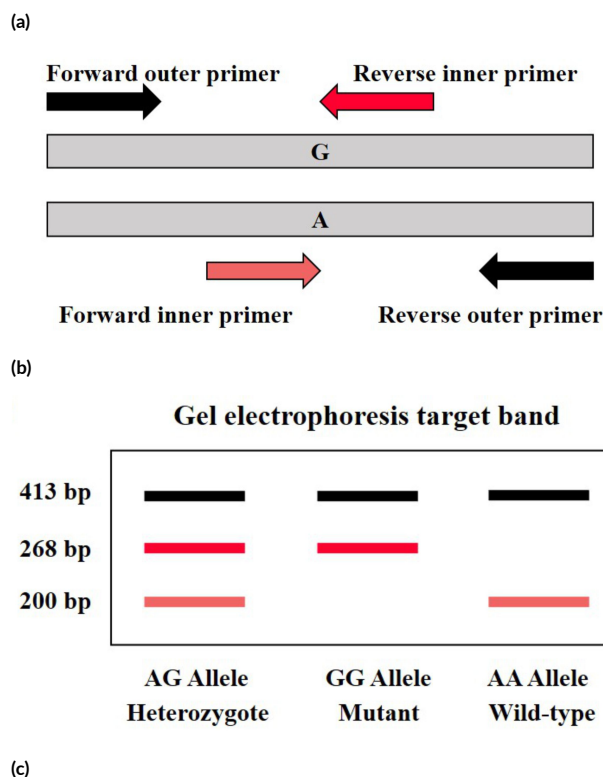

(a)

(b)

(c)

FIGURE 1 (a) DNA sequence used as reference for primers design was based on NCBI data taken from "Homo sapiens chromosome 15, GRCh.38.18, with reference sequence NC_000015.10" (https://www.ncbi.nlm.nih.gov/nuccore/NC_000015.10?from=78623282&to=78655586&report=genbank&strand=true). Bold font indicated codon change. (b) Illustrations on primers used in the Tetra-primer ARMS PCR. A and G indicate allele changes from adenine to guanine. (c) Illustration of *CHRNB4* rs1948 identification under in agarose gel electrophoresis.

2.3. Genotype confirmation

The found outer primers were used to amplify a *CHRNB4* gene fragment from DNA samples (C1, C2 and C3). The DNA fragment was observed using two percent agarose gel electrophoresis. Sanger DNA sequencing was used to confirm the genotype of the PCR product. The PCR product and the outer primers (30 μL each) were sent to 1st Base Sequencing Laboratories (Selangor, Malaysia) for sample processing. The sequencing outcome was examined using Poly Peak Parser software (http://yosttools.genetics.utah.edu/PolyPeakParser).

2.4. Tetra-primer ARMS PCR optimization

The tetra-primer ARMS PCR was optimized in three steps: 1) determining the optimal annealing temperature; 2) de-

termining the ratio of outer and inner primers, and 3) determining the correct mixed PCR reagents. Only two samples (C1 and C2) were used to find the optimized Tetra-primer ARMS PCR. A total of 10 µL GoTaq Green Master Mix (2X Green GoTaq Reaction Buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, and 3 mM MgCl2 (Promega Corporation, USA)) were mixed with a different ratio of outer and inner primers (each primer had working stock concentration of 10 nmol/L), sample DNA with different concentration, and Nuclease-Free Water to add up to the total volume of the PCR reaction of 20 µL. The DNA concentration for each sample was as follow: C1 = 41.89 ng/µL, C2 = 14.54 ng/µL, C3 = 13.93 ng/µL, S1 = 1.21 ng/µL, S2 = 13.93 ng/µL, S3 = 10.93 ng/µL, S4 = 3.21 ng/µL, S5 = 68.00 ng/µL, S6 = 39.50 ng/µL, S7 = 21.50 ng/µL, S8 = 19.50 ng/µL, S9 = 23.00 ng/µL, S10 = 1.70 ng/µL. Thermocycler PCR ProFlex Applied Biosystem (Life Technologies Holdings Pte, Ltd, Singapore) was used for DNA amplification.

As directed by the PCR kit, the annealing temperature was determined by lowering the average melting temperature by 5 ºC and gradually increasing by 1ºC. The annealing temperature gradients were 63.8 ºC, 64.8 ºC, 65.8 ºC, and 66.8 ºC. The chosen annealing cycles were 35. The best temperature that produced the correct band result according to the sequencing result was chosen. Tetra-primer ARMS PCR was also optimized by varying the outer and inner primer concentration ratios between 1:2, 1:3, 1:4, 1:5, 1:6, and 1:7. The optimum outer and inner primers ratio which gave a clear band with no dimers was chosen.

Additional samples were used to test the optimized PCR conditions. C3 was added to check whether the optimized tetra-primer ARMS PCR can detect the last variation (wild-type) of *CHRNB4* rs1948. Different DNA volumes were used (S1 = 1 µL, S2 = 2 µL, S3 = 3 µL, and S4 = 4 µL) to find the best volume added to the PCR reaction. Additionally, six samples were used to test the reproducibility of the optimized tetra-primer ARMS PCR. All PCR products were run in three percent agarose gel electrophoresis against the FlouroVue DNA marker (Smobio Technology, Inc. Taiwan) with 50 and 100 bp difference for visualization. GelDoc was used to visualize the images under UV transillumination (Syngene, United Kingdom).

3. Results and Discussion

Table 2 displays the minor allele frequency (MAF) of the reference population (Chinese and Malay populations that were obtained from The Singapore Genome Variation Project (https://blog.nus.edu.sg/sshsphphg/sing apore-genome-variation), Vietnamese populations were obtained from A Vietnamese Human Genetic Variation database (https://genomes.vn), and East Asian, Europe and America populations were obtained from The 1000 GENOME phase 3 project (https://www.ncbi.nlm.nih.gov /snp/rs1948#frequency_tab). Because the MAF ranged from 0.308-0.846, we concluded that the *CHRNB4* rs1948

could be detected in the Indonesian population.

With the help of Primer1, a set of outer and inner primers based on the *CHRNB4* DNA sequence which includes the rs1948 variant was designed. The final primers set for the tetra-primer ARMS PCR can be seen in Table 3. The outer primers were successfully amplified targeted DNA fragments of *CHRNB4* with 413 bp (Figure 2). Sanger sequencing confirmed the genotypes of C1 was AG allele (heterozygous mutant), C2 had GG allele (homozygous mutant) and C3 had AA allele (homozygous wild-type) (Figure 3).

Based on Figure 4a, among the four different annealing temperatures along with 1:1, 1:2, 1:3 and 1:4 primers ratio, the annealing temperature of 64.8 ºC and 35 cycles with 1:4 ratio gave the matching band as the sequencing result. As the DNA band was still unclear with primers ratio 1:4, we decided to try different ratios which were 1:5, 1:6 and 1:7. Outer and inner primers with 1:6 ratio gave the best result, so that ratio was chosen (Figure 4b).

After that, this method was used on the last variant and with different DNA volumes to get the clearer band result. We succeeded in visualizing the last variant in Figure 4c. In three percent gel electrophoresis, the final optimized Tetra-primer ARMS PCR product showed 200 bp, 268 bp, and 413 bp DNA bands for C1, 268 bp and 413 bp DNA bands for C2, and 200 bp and 413 bp DNA bands for C3. This result confirmed that the genetic variation in the C1 samples has the AG allele (heterozygous mutant), C2 samples have the GG allele (homozygous mutant), and C3 samples have the AA allele (homozygous wild-type) following the sequencing results.

We also found that using 3 µL DNA gave the best result (Figure 4c). Finally, we checked the reproducibility of this method using six different samples (Figure 4d). The tetra-primer ARMS PCR ran perfectly and all samples produced the expected band. It was determined that the Tetra-primer ARMS PCR optimization process for *CHRNB4* rs1948 detection was successful. Tables 4 and 5 describe the detailed reagents mix for tetra-primer ARMS PCR and the PCR program. Schematic illustration on how to get the
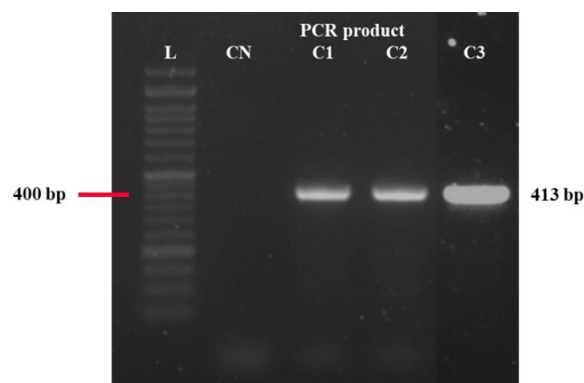

FIGURE 2 PCR product obtained by using outer primers visualized by 2% agarose gel electrophoresis showed the expected 413 bp band. (L = DNA ladder, C1 = control 1, C2 = control 2, C3 = control 3, CN = negative control).

TABLE 2 Minor allele frequency (MAF) of *CHRNB4* rs1948.

Gene Variant	Minor Allele Frequency (MAF) of Alternative Allele (G)					
	Chinese*	Malay*	Vietnam **	East Asian***	Europe***	America***
CHRNB4 rs1948	0.308	0.359	0.433	0.518	0.681	0.846

The population data were taken from *The Singapore Genome Variation Project (https://blog.nus.edu.sg/sshsphphg/singapore-genome-variation); **A Vietnamese Human Genetic Variation database (https://genomes.vn); and ***The 1000 GENOME phase 3 project (https://www.ncbi.nlm.nih.gov/snp/rs1948#frequency_tab).
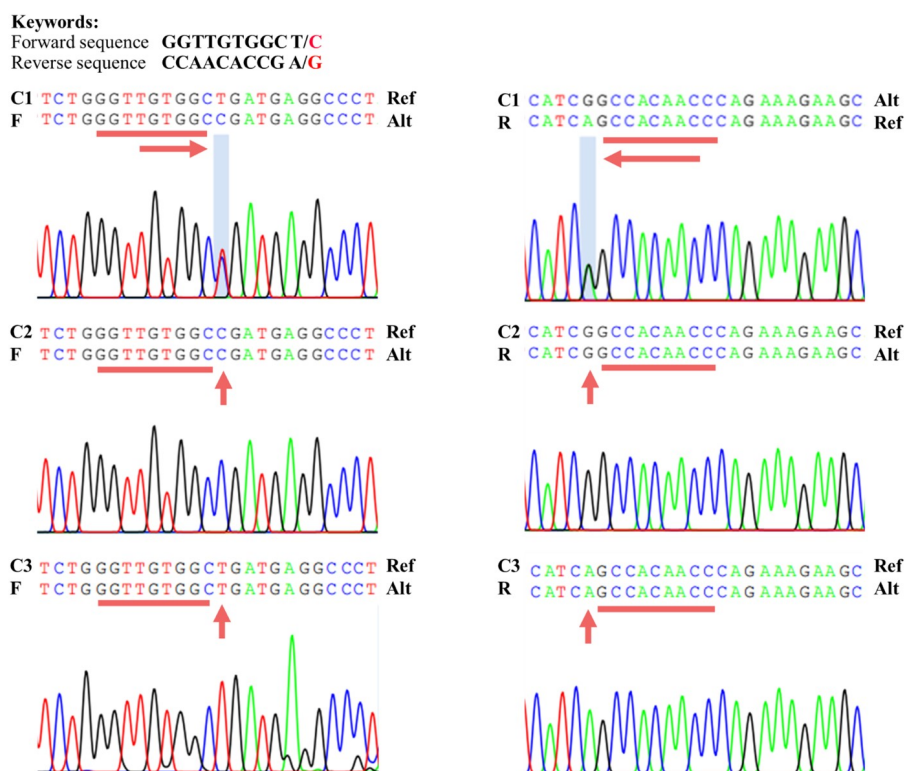

FIGURE 3 Sanger sequencing results from C1, C2 and C3 samples were analyzed using Poly Peak Parser (http://yosttools.genetics.utah.edu/PolyPeakParser). The red line showed the sequences, where the "keywords" were found. The red arrow in the C1 sample showed C codon in a forward alternative sequence and G codon in reverse sequence. The blue line indicated the heterozygous changes, thus the C1 has an AG allele. The sequencing result in the C2 sample showed C codon in a forward sequence and G codon in reverse sequence without any blue line, making the C2 sample as GG allele (mutant allele). Sanger sequencing also confirms that C3 sample showed T codon in a forward sequence and A codon in reverse sequence, which concluded C3 as AA genotype (wild-type). (C1 = control 1, C2 = control 2, C3 = control 3, F = forward sequence, R = reverse sequence, Ref = reference allele, Alt = alternative allele).

optimized tetra-primer ARMS PCR based on gel agarose electrophoresis results can be seen in Figure 4.

3.1. Discussion

Tetra-primer ARMS PCR is a genotyping method that uses two separate sets of primers, the outer and inner primers. The outer primers amplify the target gene sequence, whereas the inner primers identify the changed nucleotide in the sequences (Ye et al. 2001). This study follows the guideline for tetra-primer ARMS PCR optimization from Medrano and De Oliveira (2014).

The use of Primer1, a free website for designing primers for Tetra-primer ARMS PCR, was in accordance with Collins and Ke (2012). Primer design is a critical step in ensuring the success of the Tetra-primer ARMS PCR

Medrano and De Oliveira (2014); Zabala et al. (2017). A successful primer set depends on many variables including melting temperature (Tm), total GC content, and selection of mismatch bases (Collins and Ke 2012). Zabala et al. (2017) also stated that the interaction of the inner and outer primers may affect the specificity. Using the Primer1 web service, we can get the optimal inner primer pair that matches the closest input Tm and the minimum Tm difference between the pairings. Outer primer pairs selected should be matched to the Tm mean of the inner primers. Furthermore, Primer1 will also add a mismatched base at the 3' terminus which will increase the specificity of allele detection (Ye et al. 2001; Suhda et al. 2016).

With the correct input, Primer1 will serve 10 different primer sets and their respected DNA products. According
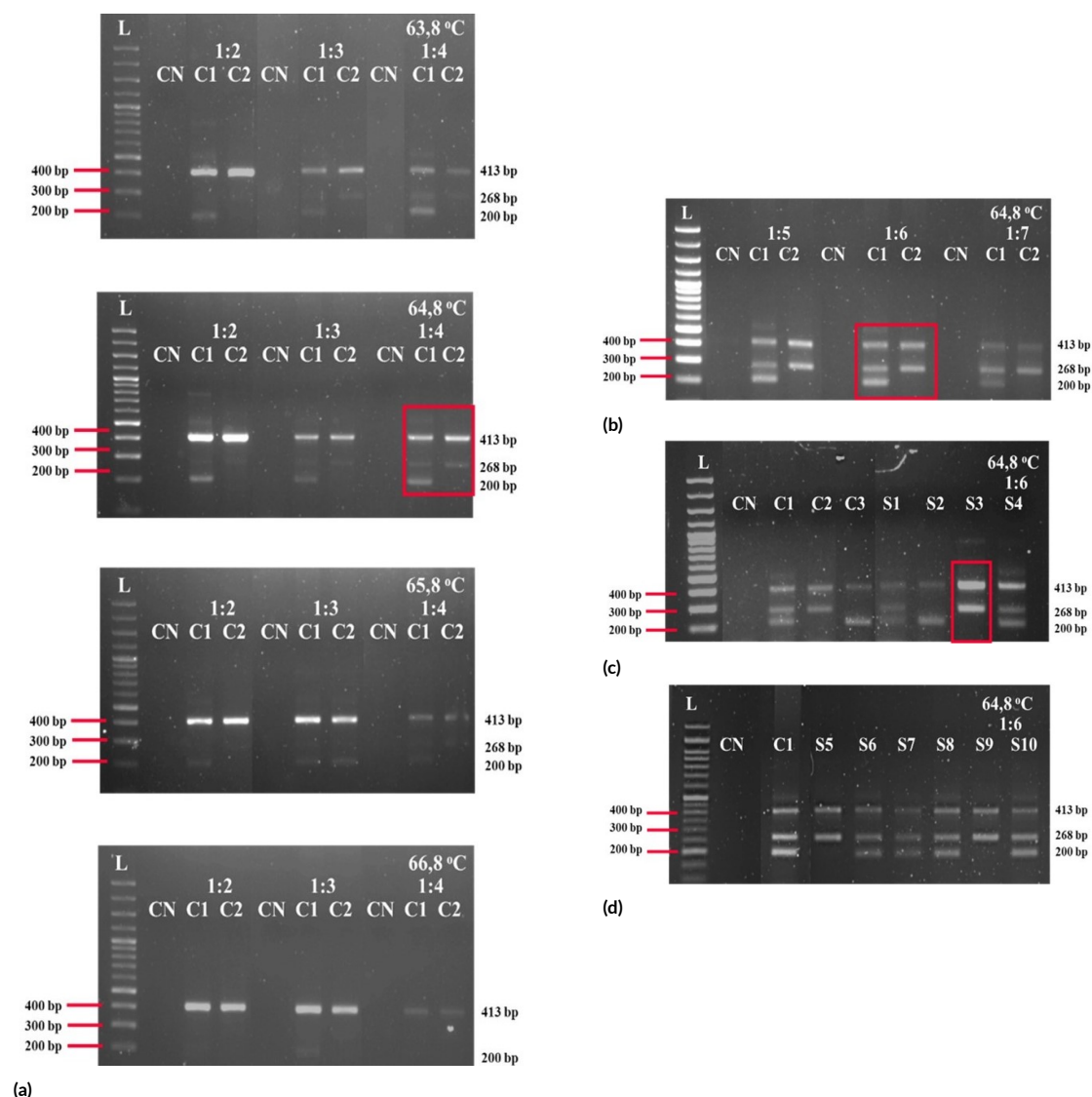
FIGURE 4 Optimization process of tetra-primer ARMS PCR to detect *CHRNB4* rs1948. PCR products run in 3% of agarose gel electrophoresis. (a) PCR with the variation of annealing temperatures of 63.8 °C, 64.8 °C, 65.8 °C, and 66.8 °C with outer and inner primers ratio of 1:2, 1:3, and 1:4 (see the label in each picture); all done in 35x PCR cycles. Results showed annealing temperature of 64.8 °C as the optimum PCR condition, which shown the clearest AG genotype in C1 sample and GG genotype in C2 sample. (b) Further optimization of outer and inner primer ratios showed 1:6 as the best, beside 1:5 and 1:7. To conclude, optimization with 1:6 of outer and inner primer ratio in 64.8 °C annealing temperatures with 35x annealing cycle showed the best result with minimum primer dimers. (c) The last variant was checked and found that the method can produce the AA genotype in C3 sample. Different volume of DNA (S1 = 1 μL (1.21 ng DNA), S2 = 2 μL (13.93 ng DNA), S3 = 3 μL (10.93 ng DNA), and S4 = 4 μL (3.21 ng DNA)) were used to get the clearer band results. A DNA volume of 3 μl was chosen. (d) Reproducibility test of the Tetra-primer ARMS PCR in six different samples (S5-S10; S5 = 68.00 ng/μL, S6 = 39.5 ng/μL, S7 = 21.5 ng/μL, S8 = 19.5 ng/μL, S9 = 23.00 ng/μL, S10 = 1.70 ng/μL). (L = DNA ladder, CN = negative control, C1 = control 1, C2 = control 2, S1 = sample 1, S2 = sample 2, S3 = sample 3, S4 = sample 4, S5 = sample 5, S6 = sample 6, S7 = sample 7, S8 = sample 8, S9 = sample 9, and S10 = sample 10).

to Suhda et al. (2016), to achieve good visualization under UV transillumination, it is important to choose primer sets that have a target band difference with a minimum of 50 bp, since the DNA bands could easily be separated by 2-3% agarose gel electrophoresis. Thus, we chose primer sets with 413 bp, 268 bp, and 200 bp products.

For the optimizing step, a PCR machine with a gradient PCR system would allow different annealing temperatures to be applied in the same PCR process. Ye et al. (2001) proposed that the optimum annealing temperature of 5°C lower than the average melting temperature to pro-

duce the most stable PCR product. The Tm mean in the primers that we used was 68.775 °C (∼68.8 °C). We applied annealing temperatures from 63.8 oC and gradually increased by increments of 1 °C. We identified that 64.8 °C yielded the best product, close to suggestion by Ye et al. (2001). On the other hand, Medrano and De Oliveira (2014) and Suhda et al. (2016) discovered that the optimum annealing temperature was closer to the average melting temperature. We concluded that different genetic variants may produce different results; thus, optimization with a wide range of temperatures is still needed.

TABLE 3 Primers used in tetra primer ARMS PCR genotyping.

Variant	Primary sequence	Product (bp)	Tm (°C)
CHRNB4 rs1948	*Forward outer primer (5' – 3'):* CTGCAGGTCGTTGAGGACTG-GAAGTA	413	69.5
	Reverse outer primer (5' – 3'): GAAGACAGGCCA-GAATTGAACTGTCTGA	413	68.7
	Forward inner primer (Allele A): TTTGCT-GCTTCTTTCTGGGTTGTGTCT	200	66.8
	Reverse inner primer (Allele G): GCTCACATATTTACTTAGGGC-CTCAGCG	268	70.1

*Primers were designed by using Primer1 (http://primer1.soton.ac.uk/public_html/primer1.html)

TABLE 4 Reagent mix for tetra-primer ARMS PCR *CHRNB4* rs1948.

Reagent	Working concentration	Volume
GoTaq® Green Master Mix		10 µL
Nuclease Free Water		4.2 µL
Forward outer primer	10 nM	0.2 µL
Reverse outer primer	10 nM	0.2 µL
Forward inner primer	10 nM	1.2 µL
Reverse inner primer	10 nM	1.2 µL
DNA	1-70 ng/ µL*	1-4 µL
Total volume		20 µL

PCR kit: GoTaq® Green Master Mix (2X Green GoTaq® Reaction Buffer (pH 8,5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, and 3 mM MgCl2 (Promega Corporation, USA).
*Detailed concentration was C1 = 41.89 ng/µL, C2 = 14.54 ng/µL, C3 = 13.93 ng/µL, S1 = 1.21 ng/µL, S2 = 13.93 ng/µL, S3 = 10.93 ng/µL, S4 = 3.21 ng/µL, S5 = 68.00 ng/µL, S6 = 39.50 ng/µL, S7 = 21.50 ng/µL, S8 = 19.50 ng/µL, S9 = 23.00 ng/µL, S10 = 1.70 ng/µL.

TABLE 5 Tetra-primer ARMS PCR *CHRNB4* rs1948 program.

Reagent	Times	Temperature (°C)	Cycle number
Initial denaturation	2 minutes	95	1 cycle
Denaturation	30 seconds	95	
Annealing	30 seconds	64.8	35 cycles
Elongation	1 minutes	72	
Termination	5 minutes	72	1 cycle
Cooldown	∼	4	

Determining the best concentration ratio of outer and inner primer is important (Suhda et al. 2016). Having two sets of primers increases the likelihood of obtaining non-specific products and the appearance of dimers (Medrano and De Oliveira 2014). Ye et al. (2001) discovered that

decreasing the concentrations of the outer primer improves the amplification of the allele-specific product. We used 0.2 µL outer primers as a starter with the increasing inner primers, following the ratio of 1:2, 1:3, 1:4, 1:5, 1:6, and 1:7. In accordance with Ye et al. (2001), we also found that lowering the outer primers concentration truly gives the expected target band result under trans UV illumination.

We observed that initial DNA concentration often did not match their respective DNA band under agarose gel electrophoresis. Because we used DNA with low concentration, we tried to get a clear band without using too much DNA. Thus, we tried to use different DNA volume start from the lowest. Our final check on the optimized tetra-primer ARMS PCR confirmed that 3 µL instead of 1 µL showed the best product when ran under agarose gel electrophoresis. Previous study also tried to use 1-3 µL (Suhda et al. 2016). Different DNA quality may give low reproducibility (Medrano and De Oliveira 2014). We also got a different band result than the controls, which was later confirmed to have an AA allele (homozygous wild-type), consistent with our findings using the optimized tetra-primer ARMS PCR.

Finally, within this report we could identify three alleles of *CHRNB4* rs1948 during the optimization of genotyping method employing the tetra-primer ARMS PCR, as confirmed by Sanger sequencing. We are planning to use the optimized method to analyze the association of genetic polymorphism, tobacco smokes exposure, and risk of cancer.

4. Conclusions

We successfully developed the tetra-primer ARMS PCR protocol for *CHRNB4* rs1948 genotyping. Furthermore, the novel tetra-primer ARMS PCR may be useful for large-scale association studies based on different SNV using only a conventional PCR machine.

Acknowledgments

Authors' contributions

JF, ALD, DK designed the study. ALD conducted the laboratory work. ALD, DK, JF analyzed the data. ALD and JF wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no conflict of interest concerning the present paper.

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