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Method Development of Nested Allele-Specific Multiplex Polymerase Chain Reaction (NASM-PCR) for the Irritable Bowel Syndrome (IBS)-related Gene Polymorphisms

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Abstract

Polymorphisms in the genes of G-protein subunit beta 3 (*GNB3*); rs5443, tryptophan hydroxylase 1 (*TPH1*); rs211105 and rs4537731, tryptophan hydroxylase 2 (*TPH2*); rs4570625 and sodium voltage-gated channel alpha subunit 5 (*SCN5A*); rs1805124, have known to cause the abnormalities in the gastrointestinal tract that are implicated to irritable bowel syndrome (IBS) predisposition. Upfront genetic polymorphism genotyping in IBS-related gene polymorphisms will help to intervene and guide the decision-making in the management of IBS patients. This study aimed to develop a genotyping method to detect the respective polymorphisms using nested allele-specific multiplex polymerase chain reaction (NASM-PCR). A combination of nested and allele-specific multiplex PCR method was developed to determine the five single nucleotide polymorphisms (SNPs) mentioned. Annealing temperature, annealing and extension times, and the concentrations of $MgCl_2$, primers, and DNA samples were optimized in the PCR. Sanger sequencing was performed to validate the genotyping results. NASM-PCR for IBS-related gene polymorphisms were successfully developed. DNA bands which correspond to the genes and SNPs have shown 100% homologous with the gene database. The developed method of NASM-PCR was reproducible and specific to be used for determining the respective polymorphisms of IBS. Notably, the described method can easily be integrated into other laboratories for population study or clinical use.

Keywords: Irritable bowel syndrome (IBS), gastrointestinal (GI) tract, nested allele-specific multiplex PCR, genotyping method, single nucleotide polymorphism (SNP).

1. Introduction

Functional gastrointestinal disorders (FGIDs) are chronic or recurrent gastrointestinal conditions which perceived no structural or biochemical symptoms. The FGIDs may not a life-threatening issue, but their consequences severely impact the quality of life as the problems are highly prevalent, impair routine tasks, and inflict great socioeconomic burden [1,2,3]. Sharing similar typical pathophysiological mechanisms with the functional dyspepsia

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(FD) in terms of sleep disturbance, visceral hypersensitivity, impaired central processing of sensory perception, gastrointestinal (GI) dysmotility, and psychological circumstances; irritable bowel syndrome (IBS) is known as the most typical FGIDs symptoms with high prevalence rates worldwide [4,5].

Polymorphisms in G-protein subunit beta 3 (*GNB3* gene*; rs5443*), Tryptophan hydroxylase 1 (*TPH1* gene*; rs211105 & rs4537731*), Tryptophan hydroxylase 2 (*TPH2* gene*; rs4570625*) and Sodium voltage-gated channel alpha subunit 5 (*SCN5A* gene*; rs1805124*) are known to be associated with the IBS [6,7,8,9]. Due to the presence of the polymorphisms, many protein functions encoded by these genes, including the ion channel, motility, secretion, and contraction, were disrupted, resulting in physiological symptoms such as diarrhea, bloating, and loose stools [10,11,12]. Because genetic variants are strongly linked to many clinical phenotypes, investigating the IBS-related gene polymorphisms will aid in intervening and guiding patient care decisions [13].

Numerous polymerase chain reaction (PCR) genotyping methods have been rapidly invented in response to the detection of genetic variations for susceptibility to human diseases and personalised therapy. However, most of new, high-performance, automated genotyping analysis systems are expensive or require specific tools and are occasionally incompatible with traditional laboratory settings. Nested allele-specific multiplex PCR (NASM-PCR) is one of the most common genotyping procedures used to detect SNPs since it is inexpensive, simple to use and delivers accurate findings [14]. In addition, it is more feasible and appropriate for the large-scale genotyping study too [13,14]. Therefore, the objective of this study is to develop a NASM-PCR genotyping method to detect the IBS-related gene polymorphisms. The study's developed method can be used to establish a genotype database of population-/geographic-specific research in order to explore the genotype-phenotype relationship of IBS and other functional gastrointestinal medical conditions interindividually.

2. Materials and methods

2.1 Source of samples

In the study, archived DNA samples from the blood were occupied during the genotyping method development. Forty-eight blood samples of unrelated and healthy Malay volunteers were randomly chosen from a previous study, Development of ethno-pharmacogenetics relatedness and personalized medicine (Grant No. 1001/PSK/8620013). The human ethical approvals were obtained from the Universiti Sultan Zainal Abidin (UniSZA) Human Research Ethics Committee (UHREC), Terengganu, Malaysia (Reference number: UniSZA.C/2/UHREC/628-2(73)) and the Human Research Ethics Committee (HREC), Universiti Sains Malaysia (USM) in Kelantan, Malaysia (Reference number: USM/JEPeM/19020149).

2.2 DNA extraction

The DNA was extracted from whole blood using QIAamp® DNA Blood Mini Kit (QIAGEN, Hilden, Germany), according to the standard protocol by the manufacturer. The DNA concentration and integrity were determined using Nanodrop ND-1000 spectrophotometer (Wilmington, USA) and 1% agarose gel electrophoresis, respectively.

2.3 Primers design

Primers of the interest genes; *GNB3*, *TPH1*, *TPH2* and *SCN5A* and its single nucleotide polymorphisms; rs5443, rs211105, rs4537731, rs4570625 and rs1805124 with specific 3’-end for polymorphic sites were designed based on the latest gene sequence from the GenBank database. The primers in the first PCR (Table 1) were designed to amplify a specific area in GNB3, TPH1, TPH2, and SCN5A genes of interest. This was followed by a second PCR

employing primers (Table 2) that were modified to distinguish single nucleotide alterations at specific loci during PCR amplification. The sequences were checked using Primer3 and their specificity was validated by PrimerBLAST. Tables 1 and 2 exhibit the list of the designed primers used in the study.

Table 1: List of primers used in PCR1 of NASM-PCR. The primers have been appended with 'FW' and 'RV' for forward and reverse primers followed by the gene's specific name for ease of reference.

Gene	Primer Name	Primer sequence	Product size (bp)
GNB3	FW_GNB3 RV_GNB3	CTG ATC CCT GAC CCA CTT GC AGT CCG AAA TGG GAG CTG A	349
TPH1	FW_TPH 1A RV_TPH 1A	AGG ACT GTA CAC ATA ACG AAG TAT TGA AAG GTC TCT CCC TGA CCA	664
TPH1	FW_TPH 1B RV_TPH 1B	AAC CAA GGA ACA GTT TCC ATA CCT AAA CAG AAG GGT AGG GTG GG	571
TPH2	FW_TPH 2 RV_TPH 2	GCT TTC TCC TCA CCA CAT AAC G CTG GCA AGT TAA CCT CAG TCT	801
SCN5A	FW_SCN5A RV_SCN5A	GGG TGC TCT AGC ATC ACA GG GAT GAA AAC AGC ACA GCG GG	245

Abbreviations: *TPH1*; Tryptophan hydroxylase 1 gene, *TPH2*; Tryptophan hydroxylase 2 gene, *SCN5A*; Sodium Voltage-Gated Channel Alpha Subunit 5 gene, *GNB3*; G protein subunit beta 3 gene

Table 2: List of primers used in PCR2 of NASM-PCR. The primers have been appended with 'FW' and 'RV' for forward and reverse primers for ease of reference. Notably, each set of primers consists of PCR1 and newly designed primers to exhibit specific nucleotide (in bold) of wild or mutant type variant of the specific SNP at the 3'-end of allele specific primer.

SNP	Primer name	Type	Primer sequence	Product size (bp)
rs5443 (10501C>T)	FW_rs5443C RV_GNB3	Wild	TCA TCT GCG GCA TCA CGT C**C** AGT CCG AAA TGG GAG CTG A	208
	FW_rs5443T RV_GNB3	Variant	TCA TCT GCG GCA TCA CGT C**T** AGT CCG AAA TGG GAG CTG A	
rs211105 (18033757T>G)	FW_ TPH 1B RV_rs211105A	Wild	AAC CAA GGA ACA GTT TCC ATA CCT GAT TTC TAA GAT CTT TTC CAT CGG C**A**	430
	FW_ TPH 1B RV_rs211105C	Variant	AAC CAA GGA ACA GTT TCC ATA CCT GAT TTC TAA GAT CTT TTC CAT CGG C**C**	
rs4537731 (18047335T>C)	FW_rs4537731T RV_ TPH 1A	Wild	TGG ATG TAC TTT AAA GCT CAG GA**T** TGA AAG GTC TCT CCC TGA CCA	251
	FW_rs4537731C RV_ TPH 1A	Variant	TGG ATG TAC TTT AAA GCT CAG GA**C** TGA AAG GTC TCT CCC TGA CCA	
rs4570625 (4298G>T)	FW_ TPH2 RV_rs4570625G	Wild	GCT TTC TCC TCA CCA CAT AAC G AGC TTT TTC TGA CTT GAC ATA TT**C**	390
	FW_ TPH2 RV_rs4570625T	Variant	GCT TTC TCC TCA CCA CAT AAC G AGC TTT TTC TGA CTT GAC ATA TT**A**	
rs1805124 (50744A>G)	FW_ SCN5A RV_rs1805124A	Wild	GGG TGC TCT AGC ATC ACA GG GGA GAG CGA GAG CCA CC**A**	225
	FW_ SCN5A RV_rs1805124G	Variant	GGG TGC TCT AGC ATC ACA GG GGA GAG CGA GAG CCA CC**G**	

Abbreviations: SNP; single nucleotide polymorphism, **N**; substitution allele of the SNP, rs4537731; SNP of *TPH 1*, rs211105; SNP of *TPH 1*, rs4570625; SNP of *TPH 2*, rs1805124; SNP of *SCN5A*, rs5443; SNP of *GNB3*

2.4 Development of first and second PCRs (PCR1 & PCR2)

In the study, a two-step PCR combining of the nested and allele-specific multiplex method were developed; PCR1 and PCR2. For PCR1, specific region in the genes; *GNB3*, *TPH1*, *TPH2* and *SCN5A* were amplified. The amplifications from the PCR1 were used as the template for PCR2. The temperature gradient was performed for each set of primers of PCR1 and 2 to obtain the optimum annealing temperature (T_a). The temperature range tested was based on the primer melting temperature (T_m). Other factors such as annealing and extension times, the concentration of $MgCl_2$, and primers were also evaluated sequentially based on the previous method [15,16]. The PCR reactions were carried out using Eppendorf thermal cycler (Eppendorf AG, Hamburg, Germany). All PCR reagents and chemicals used were from Promega (Wisconsin, USA). To improve the PCR electrophoresis result, the agarose gel was also optimized to obtain a seamless band as seen in Plates 1 and 2.

2.5 Validation of PCR specificity by DNA sequencing

Several selected samples from PCR1 and 2 were purified using QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and bi-directionally DNA sequencing was performed using BigDye® Terminator V3.1 Cycle Sequencing Kits (Applied Biosystems, California, USA) according to the manufacturer's instructions. The purified DNA was run in 3130 Genetic Analyzer (Applied Biosystems, California, USA) before the results were analyzed using Chromas Lite Version 2.01 (Technelysium, Queensland, Australia). The bi-directional sequencing result was subjected to the Basic Local Alignment Tool (BLAST) program of NCBI for comparison of the sequences to identify the accuracy and efficiency of the developed NASM-PCR method. The PCR template sequences should be greater than 95% homologous to the GenBank database to meet the criteria of specificity. Chromatogram results were also inspected for any heterozygosity variants to build as a positive control in the future batch of the PCR.

3. Results and discussions

A NASM-PCR method was successfully developed to detect *GNB3*, *TPH1*, *TPH2* and *SCN5A* and five SNPs; rs5443, rs211105, rs4537731, rs4570625 and rs1805124. The development of the IBS-related gene polymorphisms PCR method in this study was designed with the combination of nested, multiplex and allele specific techniques. Nested PCR helps to increase the sensitivity and specificity in amplifying the DNA samples using two primer sets with two successive PCR reactions [17,18]. While the PCR2.B and C were manipulated from the multiplex PCR, a widespread molecular technique which can simultaneously amplify two or more target sequences in a single reaction tube [21]. The allele specific PCR allows the amplification of the SNPs only if the 3'-end of the wild-type or variant primers was thoroughly complemented, therefore it would reduce the false-positive genotyping result [20,21]. The presence of bands at the correct size on the gel electrophoresis indicated successful PCR amplification. The incorporation of nested, multiplex and allele-specific PCR for the IBS-related gene polymorphisms have produced a less cost- and time-consuming genotyping method.

3.1 The optimization of PCR1 and PCR2

All parameters such as the T_a, annealing and extension times, concentration of $MgCl_2$, primers and DNA samples were successfully optimized. Primers used in PCR1 and 2 were

shown in Tables 1 and 2. All respective genes were amplified simultaneously in the PCR1. The primers in the PCR2 are able to amplify efficiently and enrich the specific template using the amplification of the PCR1. For PCR2, it is recommended to use a diluted concentration of the PCR1's products due to the enhancement of amplifications, therefore, only 2µL over 1:20 dilutions from PCR1's products were administered in the PCR2 of the study. In the PCR1, 4µL of 10ng/µL DNA sample was used for each reaction mixture.

The optimized PCR ingredients of 1 and 2 were shown in Tables 3 and 4 respectively. Basically, the PCR mixture was split into two parts; Master mix and Primer mix. The master and the primer mixes were combined in a PCR tube before the required amount of DNA sample was added at the final stage of preparation. The optimized thermal cycling conditions of PCR1 and 2 are summarized in Table 5. Each PCR1 product was extended to PCR2 for further SNPs determination. While for PCR2, it was separated into three reactions for the amplification of rs1805124 in A, rs211105 and rs5443 in B while rs4570625 and rs4537731T in C. Each sample was projected to two sets of primers; wild and variant types to determine the heterozygosity type variant.

Table 3: The optimized PCR ingredients for PCR1. Each reaction mixture was prepared for 25µL with 4µL of DNA sample (10ng/µL) added. The initial stock primer concentration was 5µM.

PCR ingredients	Volume per reaction (µL)
Master mix (9µL)	
dH2O	1.3
1X PCR Buffer (5X)	5.0
2.0mM $MgCl_2$ (25mM)	2.0
0.2mM dNTPs (10mM)	0.5
1.0U *Taq* polymerase (5U)	0.2
Primer mix (12µL)	
0.25µM FW_GNB3	1.25
0.25µM RV_GNB3	1.25
0.25µM FW_TPH1A	1.25
0.25µM RV_TPH1A	1.25
0.20µM FW_TPH1B	1.00
0.20µM RV_TPH1B	1.00
0.25µM FW_TPH2	1.25
0.25µM RV_TPH2	1.25
0.25µM FW_SCN5A	1.25
0.25µM RV_ SCN5A	1.25

Table 4: The optimized PCR ingredients for PCR2. The reaction mixture was prepared for a total volume of 25µL with 2µL of diluted 1st PCR product (1:20) was added per reaction. Primers of PCR2 were designed specifically to match specific polymorphic sites. The initial stock primer concentration was 5µM.

PCR Ingredients	Volume per reaction (µL)/ Final concentration		
	A	**B**	**C**
Master mix (10µL)			
dH_2O	2.8	2.3	3.3
PCR Buffer (5X)	5.0/ 1X	5.0/ 1X	5.0/ 1X
$MgCl_2$ (25mM)	1.5/ 1.5mM	2.0/ 2.0mM	1.0/ 1.0mM
dNTPs (10mM)	0.5/ 0.2mM	0.5/ 0.2mM	0.5/ 0.2mM
Taq polymerase (5U)	0.2/ 1.0U	0.2/1.0U	0.2/ 1.0U
Primer mix (Wild type, 13µL)			
dH_2O	12.0	8	8.5
FW_SCN5A	0.5/ 0.1µM	-	-
RV_rs1805124A	0.5/ 0.1µM	-	-
FW_ TPH1B	-	2.0/ 0.4µM	-
RV_rs211105T	-	2.0/ 0.4µM	-
FW_rs5443C	-	0.5/ 0.1µM	-
RV_GNB3	-	0.5/ 0.1µM	-
FW_ TPH2	-	-	1.5/ 0.3µM
RV_rs4570625G	-	-	1.5/ 0.3µM
FW_rs4537731T	-	-	0.75/ 1.5µM
RV_ TPH 1A	-	-	0.75/ 1.5µM
Primer mix (Variant type, 13µL)			
dH_2O	12.0	8	8.5
FW_SCN5A	0.5/ 0.1µM	-	-
RV_rs1805124G	0.5/ 0.1µM	-	-
FW_ TPH1B	-	2.0/ 0.4µM	-
RV_rs211105G	-	2.0/ 0.4µM	-
FW_rs5443T	-	0.5/ 0.1µM	-
RV_GNB3	-	0.5/ 0.1µM	-
FW_ TPH2	-	-	1.5/ 0.3µM
RV_rs4570625T	-	-	1.5/ 0.3µM
FW_rs4537731C	-	-	0.75/ 1.5µM
RV_TPH1A	-	-	0.75/ 1.5µM

Table 5:The optimized thermal cycling conditions for PCR 1 and 2. The lids of the thermal cycler were sustained at 105°C before its cycling performance began.

PCR profile	PCR1			PCR 2					
				A/B			**C**		
	Temp. (°C)	Time	Cycles	Temp. (°C)	Time	Cycles	Temp. (°C)	Time	Cycles
Pre-denaturation	95	5 min	1	95	2 min	1	95	2 min	1
Denaturation	95	30 sec	34	95	30 sec	20	95	30 sec	20
Annealing	65	30 sec		68	30 sec		62	30 sec	
Extension	72	1 min		72	30 sec		72	30 sec	
Final extension	72	5 min	1	72	5 min	1	72	5 min	1
Hold	12	∞	-	12	∞	-	12	∞	-

3.3 Genotypic and Allelic Frequencies

Plate 1 shows the amplifications of the selected region in *GNB3*, *TPH1* (divided into two sequences, A and B), *TPH2* and *SCN5A* genes using the condition of the PCR1 (Table 5). Plate 2 (A, B and C) exhibits the amplification of respective SNPs using the PCR2 thermal cycling conditions; A, B and C respectively. The PCR2 demonstrated the heterogeneity of the polymorphisms according to the oneself which was amplified initially in PCR1. When the individual does not have a variant nucleotide (mutant allele), there'll be no amplification in the mutant lane, therefore the sample was reported as homozygous wild-type. The sample was categorized as heterozygous if both lanes (wild and mutant) showed bands, whereas homozygous mutant if only one band showed in the mutant lane. All 48 Malay samples were successfully genotyped for the IBS polymorphisms (Table 6). Data obtained was only the preliminary result and no extrapolation to the population SNP frequencies were attempted at this stage.

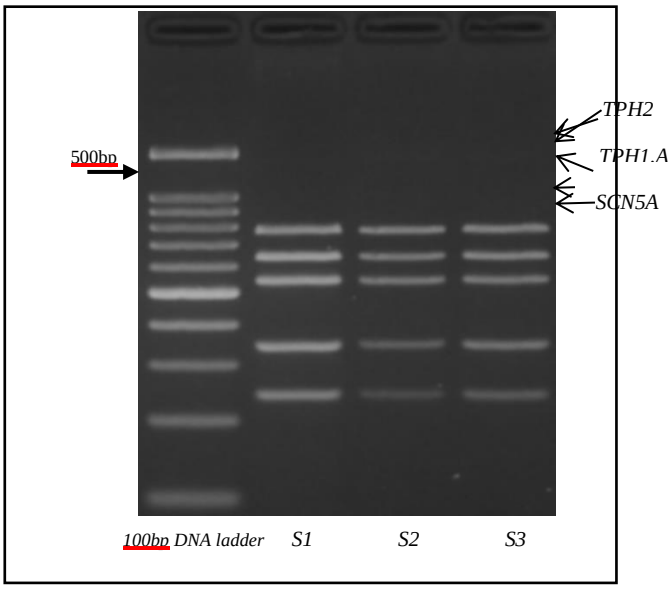

Plate 1-Amplification bands of the interest genes in PCR1. The amplification size of *TPH2* was 801bp, *THPH1.A* was 664bp, *THPH1.B* was 571bp, *GNB3* was 349bp and *SCN5A* was 245bp. For PCR1, the amplified products were electrophoresed on an ethidium bromide-stained 1% (w/v) agarose gel. ***Abbreviations***: bp: base pair, *Sn*: Subject number.

Table 6: Genotype and allele frequencies of 48 unknown healthy Malays.
No interpretation of the population was made from this data.

SNP	N	Prevalence, %	Allele	Frequency
rs5443 (C>T)				
C_C	20	41.67	C	0.604
C_T	18	37.50	T	0.396
T_T	10	20.83		
rs211105 (T>G)				
G_G	9	18.75	T	0.635
T_G	17	35.42	G	0.365
T_T	22	45.83		
rs4537731 (T>C)				
C_C	10	20.83	T	0.604
T_C	18	37.50	C	0.396
T_T	20	41.67		
rs4570625 (G>T)				
G_G	33	68.75	G	0.833
G_T	14	29.17	T	0.167
T_T	1	2.08		
rs1805124 (A>G)				
A_A	35	72.92%	A	0.854
A_G	12	25.00%	G	0.146
G_G	1	2.08%		

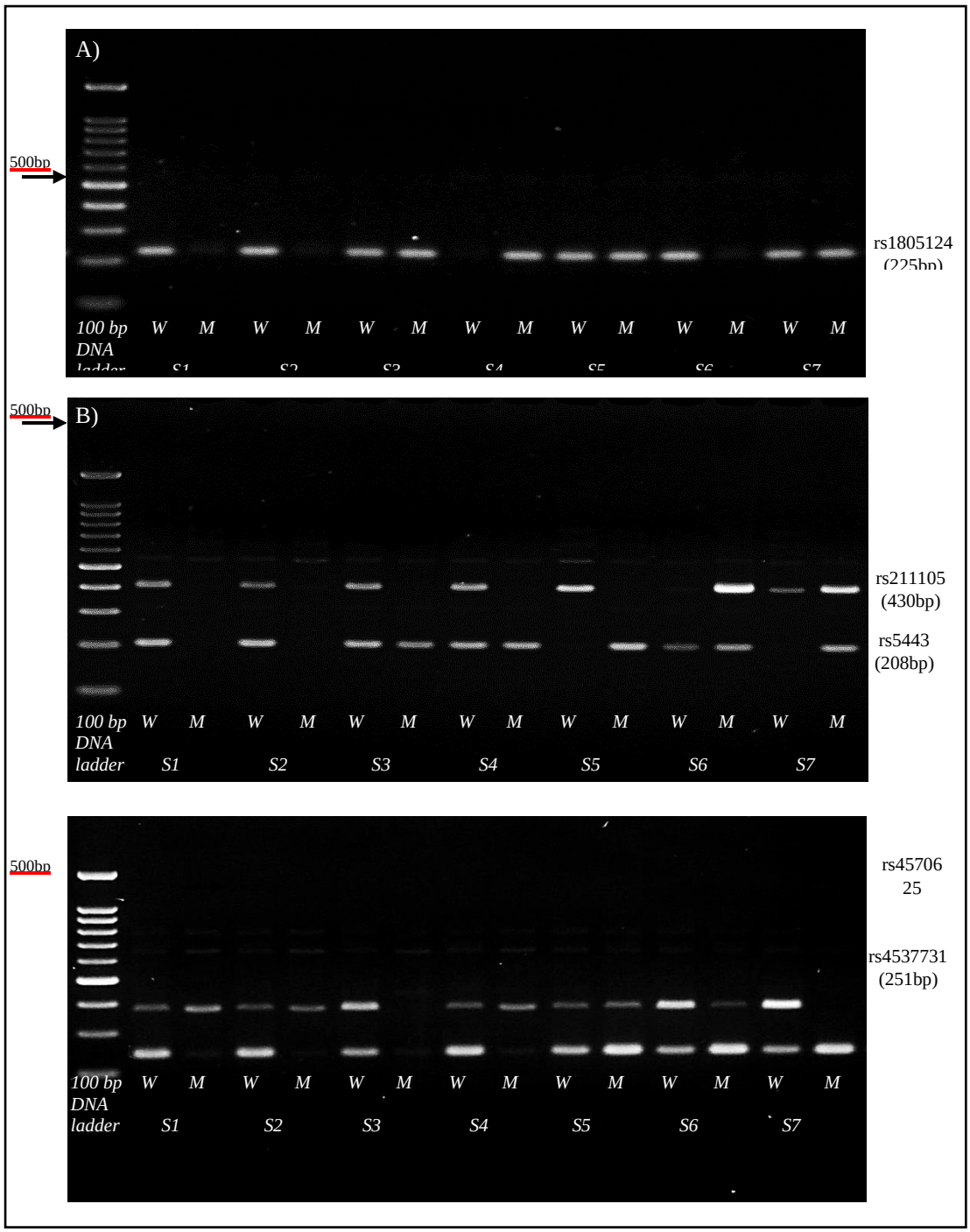

Plate 2-Amplification bands of the interest SNPs in PCR2. The amplified products were electrophoresed on an ethidium bromide-stained 2% (w/v) agarose gel. The occurrence of blurry nonspecific bands between 500 to 600bp in PCR2.B did not compensate the actual SNP readings. ***Abbreviations***: *W*: Well consisted of wild type primer set, *M*: Well consisted of variant type primer set, *Sn*: Subject number

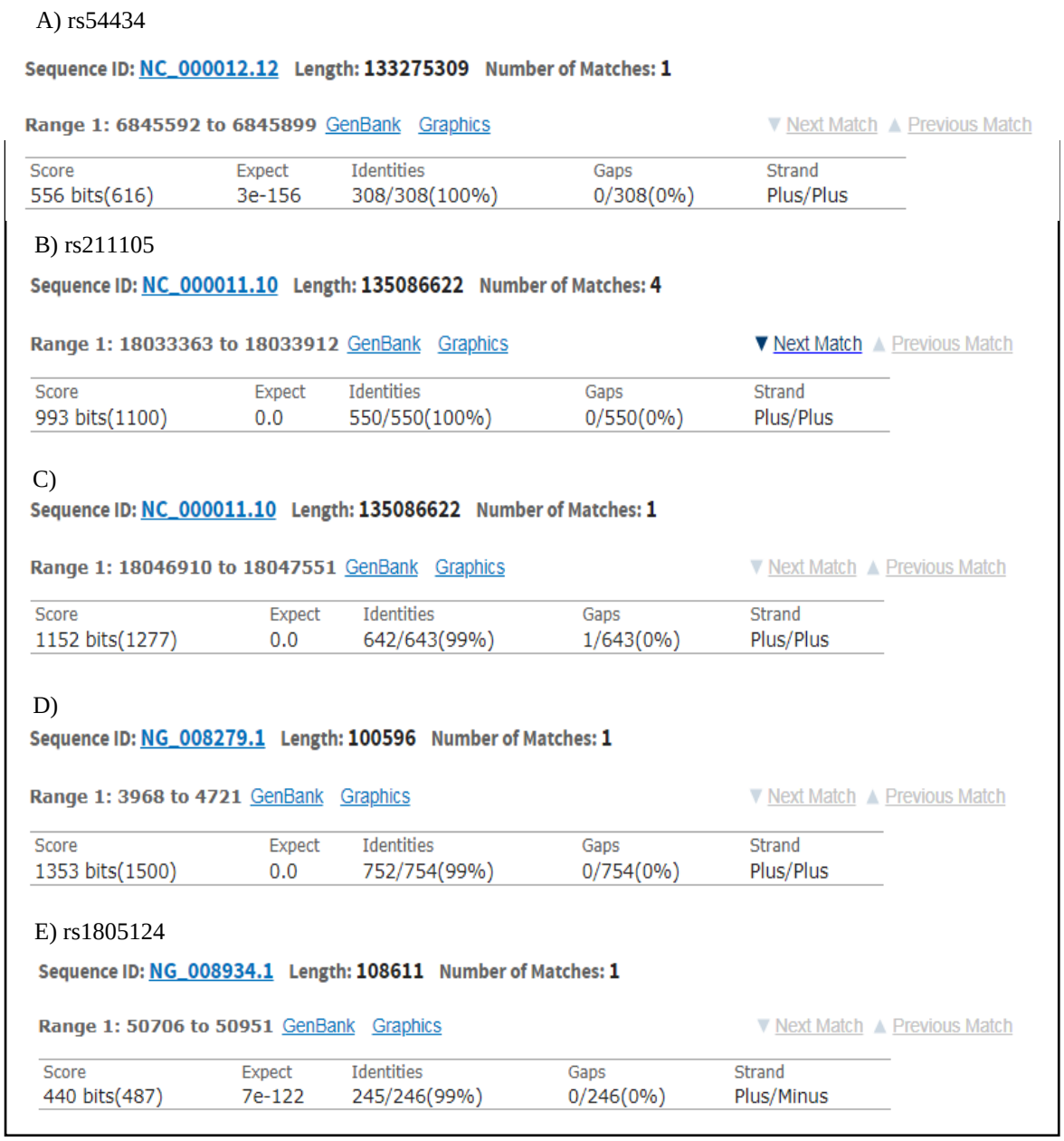

Figure 1: Amplified sequences after being subjected to BLAST exhibited completely identical results. The 99% homolog occurred when the sequence consists of mutant allele. The accuracy evaluation of developed NASM-PCR was assessed by comparison with the GenBank database in corresponding to the accession number of the SNP.

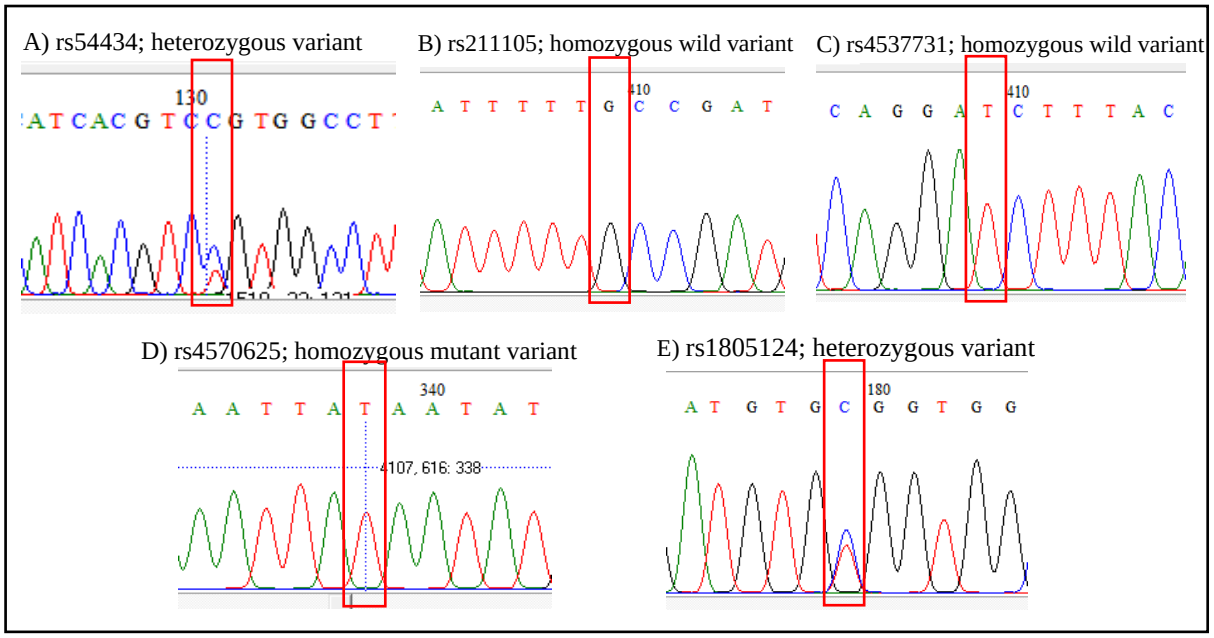

Figure 2: Chromatogram from Sanger sequencing as validation tool for genotyping result of NASM-PCR method

3.4 Validation and Confirmation Result

The quality assessment and comparison of genotype calls obtained in final genotyping set of 48 samples NASM-PCR showed high reproducibility and robustness by having 100% overall efficiency as can be seen in Figure 1. Based on the Sanger sequencing results (Figure 2), the heterozygosity types of the SNPs were compliant.

5. Conclusion

Genetic polymorphisms have been proven to be correlated with the onset and progression of IBS worldwide. The drawbacks have been recurrently noticed and varied among different ethnic variations. Thus, in-depth discussion and evaluation on the IBS-related gene polymorphisms are urgently required to better comprehend the genetic risk profiling of FGIDs, especially the IBS. This study has successfully developed a simple and specific method for IBS-related SNPs; rs5443, rs211105, rs4537731, rs4570625 and rs1805124 via NASM-PCR. The method is highly desirable for large scale genotype-phenotype FGID correlation in population studies or clinical diagnostic analyses. It is also economical and envisaged to be explored further in the future of *GNB3*, *TPH1*, *TPH2* and *SCN5A* genes.

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ETHICAL CLEARANCE

This research was ethically approved by the Universiti Sultan Zainal Abidin (UniSZA) Human Research Ethics Committee (UHREC), Terengganu, Malaysia (Reference number: UniSZA.C/2/UHREC/628-2(73)) and the Human Research Ethics Committee (HREC),

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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