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Development of PCR Method for the Detection of mu Opiate Receptor Polymorphism

Dissertation submitted in partial fulfillment for the
Degree of Bachelor of Science (Forensic Science)

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CERTIFICATE

This is to certify that the dissertation entitled

"Development of PCR Method for the Detection of mu Opiate Receptor Polymorphism"

is the bonafide record of research work done by

Nazila Binti Talib

during the period 23rd December 2008 to 5th May 2009

under my supervision.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS I

LIST OF TABLES IV

LIST OF FIGURES VI

LIST OF PLATES VII

LIST OF ABBREVIATION IX

LIST OF APPENDICES X

ABSTRACT 1

CHAPTER 1

INTRODUCTION 2

CHAPTER 2

REVIEW OF LITERATURE

2.1. Mu Opiate Receptor (OPRM1) 4

2.2. Genotype – Phenotype Correlation 5

2.3. Method available for the Detection of OPRM1 Polymorphism 6

2.4. Nested Allele-Specific Multiplex Polymerase Chain Reaction 8

CHAPTER 3

OBJECTIVE OF THE STUDY 10

CHAPTER 4

MATERIALS AND METHODS

4.1. Detection of Genetic Polymorphism Using Polymerase Chain Reaction 11
4.2. Chemicals, Reagents, and Instruments 11
4.3. Isolation of DNA 14
4.4. PCR-Based Genotyping 16
4.5. Reconstitution of Primers 20
4.6 Calculation of Working Solution 21
4.7. PCR-Genotyping (Optimized Method) 21
4.8. Validation 23
4.9. Gel Electrophoresis 23

CHAPTER 5

RESULTS

5.1. Isolation of DNA 27
5.2. Method Development for First PCR 28
5.3. Method Development for Second PCR 38
5.4. Validation of PCR Method 55

CHAPTER 6

DISCUSSION 57

CHAPTER 7

CONCLUSION 62

REFERENCES 63

APPENDICES 68

LIST OF TABLES

Table 4.1	SNPs in mu opiate receptor gene	11
Table 4.2	Chemicals and reagents used for PCR genotyping	12
Table 4.3	Instruments used for PCR genotyping	13
Table 4.4	List of primers of mu opiate receptor genotyping	18
Table 4.5	Standard PCR condition	19
Table 5.1	List of primers for first PCR	28
Table 5.2	Initial volume of Master Mix and Primer Mix for singlet reaction	29
Table 5.3	T_m and PCR product size for each primer in first PCR (M = calculated manually, B = basic, S = salt adjusted, and N = nearest neighbor) B, S and N calculated by using software Oligo Calc: Oligonucleotide Properties Calculator	30
Table 5.4	Cycle condition for singlet reaction in first PCR	31
Table 5.5	Initial volume of Master Mix and Primer Mix for multiplex reaction	34
Table 5.6	Cycle condition for multiplex reaction in first PCR	34
Table 5.7	Final condition of master mix and primer mix for multiplex reaction without intron 2 in first PCR	36
Table 5.8	List of primers used in second PCR	38
Table 5.9	T_m and PCR product size for each primer in second PCR. (M = calculated manually, B = basic, S = salt adjusted, and N = nearest neighbor) B, S and N calculated by using software Oligo Calc: Oligonucleotide Properties Calculator	40
Table 5.10	List of combination of appropriate SNPs at the start in multiplex reaction of second PCR	45
Table 5.11	Cycle condition for multiplex reaction in second PCR	46

Table 5.12 List of primers used, T_m, and product size for each set in multiplex reaction for second PCR after optimization 49

Table 5.13 Two different master mix was used in multiplex reaction of second PCR. Master Mix A for Set 1,2, and 3 while Master Mix B for Set 4, 5, and 6. 50

Table 5.14 Preparation of primer mix for all set in multiplex of second PCR. Each set should have two different tubes; one for wild type primers and the second one for mutant type primers 51

LIST OF FIGURES

Figure 4.1	Illustration for the site of amplification of first PCR product and second PCR product of OPRM1	17
Figure 4.2	Illustration of electrophoresis results for first PCR on 3 % agarose gel	25
Figure 4.3	Illustration of electrophoresis results for second PCR on 3 % agarose gel	26
Figure 5.1	Sequencing chromatogram had shown two peaks at position 208 for SNP IVS +691 G/C which indicates heterozygous for sample 1.	56

LIST OF PLATES

Plate 5.1	Gel electrophoresis of DNA extracted	28
Plate 5.2	Exon 1 with the product size 420 bp	32
Plate 5.3	Exon 2 with the product size 483 bp	32
Plate 5.4	Exon 3 with the product size 677 bp	33
Plate 5.5	Intron 2 with the product size 1020 bp	33
Plate 5.6	Band produced in multiplex reaction for first PCR initially	35
Plate 5.7	Multiplex reaction of exon 1, 2, and 3 with different samples	37
Plate 5.8	Singlet reaction of intron 2 with different samples	37
Plate 5.9	Singlet reaction of 17 C/T. Suitable T_m = 64.4 °C	41
Plate 5.10	Singlet reaction of 24 G/A. Suitable T_m = 64.4 °C	41
Plate 5.11	Singlet reaction of 118 A/G. Suitable T_m = 66.6 °C	41
Plate 5.12	Singlet reaction of 440 C/G. Suitable T_m = 64.4 °C	42
Plate 5.13	Singlet reaction of 454 A/G. Suitable T_m = 61.0 °C	42
Plate 5.14	Singlet reaction of 779 G/A. Suitable T_m = 64.4 °C	42
Plate 5.15	Singlet reaction of 794 G/A. Suitable T_m = 66.6 °C	43
Plate 5.16	Singlet reaction of 802 T/C. Suitable T_m = 64.4 °C	43
Plate 5.17	Singlet reaction of 820 G/A. Suitable T_m = 61.0 °C	43
Plate 5.18	Singlet reaction of 942 G/A. Suitable T_m = 64.4 °C	44
Plate 5.19	Singlet reaction of IVS2 +31 G/A. Suitable T_m = 64.4 °C	44
Plate 5.20	Singlet reaction of IVS2 +691 G/C. Suitable T_m = 66.6 °C	44
Plate 5.21	Initial multiplex reaction of Set 1, low intensity band at SNP 454 A/G	47
Plate 5.22	Initial multiplex reaction of Set 2, no PCR product for other two SNPs	47

Plate 5.23	Initial multiplex reaction of Set 3	47
Plate 5.24	Initial multiplex reaction of Set 4, no PCR products for other three SNPs	48
Plate 5.25	Initial multiplex reaction of Set 5, no PCR product produced	48
Plate 5.26	Multiplex reaction of Set 1 with different samples (Optimized set). SNP 820 G/A was clear while SNP 454 A/G was undetectable	52
Plate 5.27	Multiplex reaction of Set 2 with different samples (Optimized set). This set is acceptable.	52
Plate 5.28	Multiplex reaction of Set 3 with different samples (Optimized set). Both bands were unclear and low intensity.	53
Plate 5.29	Multiplex reaction of Set 4 with different samples (Optimized set). This set is acceptable.	53
Plate 5.30	Multiplex reaction of Set 5 with different samples (Optimized set). Very low intensity band produced on the desired band.	54
Plate 5.31	Multiplex reaction of Set 6 with different samples (Optimized set). No PCR product produced.	54
Plate 5.32	Set 2 was validated with different sample. All SNPs shown were homozygous wild type which corresponds with sequencing chromatogram.	55
Plate 5.33	Set 4 was validated with different sample. All SNPs shown were homozygous wild type except SNP IVS +691 G/C for sample 1 was heterozygous. This corresponds with sequencing chromatogram below.	56

LIST OF ABBREVIATION

°C	Celsius centigrade
µl	Microliter
Bp	Base pairs
DNA	Deoxynucleic acid
dNTP	Deoxynucleoside triphosphate
Ex	Exon
Fw	Forward
H_2O	Water
Kb	Kilo base pair
$MgCl_2$	Magnesium chloride
Min	Minute
Mt	Mutant type
PCR	Polymerase Chain Reaction
Rv	Reverse
Sec	Seconds
T_m	Melting temperature
UV	Ultraviolet
Wt	Wild Type

LIST OF APPENDICES

Appendix 1	Preparation of Stock Solution for Gel Electrophoresis	68
Appendix 2	Participants Information and Consent Form	69

ABSTRACT

Background: Mu opiate receptor serving as primary target for opiates drug. It plays a key role in addiction and pain perception. This receptor is highly polymorphic, but a simple method was not available to study its genetic polymorphism. We developed and optimized nested allele-specific multiplex PCR to detect twelve SNPs. Three of the SNPs; 118 A/G, IVS +31 G/A and IVS +691 C/G are common SNPs and have implication to human system. But others SNPs were rare SNPs and not widely studied. *Objective*: The objective of our study was to develop a simple and rapid PCR method for detecting polymorphism of mu opiate receptor (OPRM1), then, to validate the PCR method developed. *Method*: Genomic DNA was extracted from blood using Spin Protocol from QIAamp DNA mini kit. A two step PCR method was developed to detect twelve SNPs of OPRM1 gene. In the first PCR (PCR1), exon 1, 2, 3 and intron 2 of OPRM1 gene were amplified. There were two set of reaction involved in PCR1; Set 1 amplifies exon 1, 2, and 3 simultaneously while Set 2 applies for intron 2 only. The PCR products, then, were used as template in parallel allele-specific PCR (PCR2). Afterwards sequencing was used to validate the test results. *Result*: We have successfully developed and optimized PCR1 which amplified exon 1 (420 bp), exon2 (483 bp), exon 3 (677 bp), and intron 2 (1020 bp). Fortunately, only a few SNPs were able to be detected in PCR2. These SNPs consist of 24 G/A (102 bp), 440 C/G (330 bp), 802 T/C (424 bp), 942 G/A (434 bp), IVS +31G/A (162 bp), and IVS +691G/C (240 bp). Other six SNPs; 17 C/T, 118 A/G, 454 A/G, 779 G/A, 794 G/A, and 820 G/A failed to be amplified specifically. It might be due to contamination and also technique during preparation of PCR mixture. *Conclusion*: We were partially successful in developing and optimizing a multiplex PCR method which is suitable for use in population studies of OPRM1 polymorphism.

CHAPTER 1

INTRODUCTION

Generally, there are 12352 drug addicts have been found for the period of January to December 2008 (NADI, 2008). From 12352 drug addicts, 5939 are new drug addicts while the rest are repeated drug addicts. In average, there are 495 person reported as new drug addicts in a month and 534 person as repeated drug addicts. It means, there are 16 person new drug addicts and 18 person repeated drug addicts recorded per day.

Drug addiction is a complex disorder with a strong genetic component (Kreek, 1996). The gene that plays an important role in addiction is mu opiate receptor (OPRM1). This receptor is a primary target for opioid drugs and endogenous opioid peptides. Examples of opioid drugs include morphine, fentanyl, heroine, and codeine. Meanwhile endogenous peptide includes enkephalin, endorphin, and dynorphin.

In the drug abuse setting, OPRM1 was activated rapidly which results in euphoric effect. Thus, conferring the reinforcing or rewarding effects of the drug, contribute to the development of addiction. Clinical observations have suggested that individuals have varied sensitivity to opioid (Bond *et al*, 1998). It suggests potential variability in the receptor protein and gene. The variability here is the single nucleotide polymorphism (SNP). Some of the SNPs have been shown to produce profound effects on the function of the OPRM1.

For instance, SNP 118 A/G is implicated in the predisposition to drug addiction (Schinka *et al*, 2002). SNP 118 A/G causes an amino acid exchange at position 40 from asparagine to aspartate (N40D). SNP 118 A/G also affects beta-endorphin binding affinity of OPRM1 and changes the agonist potency of beta-endorphin for activating the OPRM1 (Bond *et al*, 1998). Moreover, SNP 118 A/G associates with nicotine reinforcement in woman (Ray *et al*, 2006).

Beside, combination of both mutant alleles of 118 A/G and IVS2+691 C/G (intron 2) caused opioid dependence (Szeto *et al*, 2001). In contrast, combination of both mutant alleles of 118 A/G and IVS2+31 G/A caused individual taking heroin consumed higher doses than individual who did not carry this polymorphism (Shi *et al*, 2002).

Due to functional importance of several OPRM1 SNPs, a simple and rapid method was developed to detect polymorphism of OPRM1. This PCR method acts as screening method for genetic diagnosis of SNPs in OPRM1. The method includes SNPs with reported high allelic frequencies as well as rare SNPs that may be potentially relevant. The method presented could facilitate identification of OPRM1 polymophisms with clinical relevance and thus enable individualized opioid pharmacotherapy in the future.

A simple polymerase chain reaction (PCR) method can be developed to obtain the information of OPRM1 polymorphism. The PCR method that will be developed is nested allele-specific multiplex polymerase chain reaction. A two-step nested PCR reaction was designed to detect twelve single nucleotide polymorphisms (SNP) which reside in the receptor. Those SNPS were located in the exon 1, 2, 3 and intron 2 region.

CHAPTER 2

REVIEW OF LITERATURE

2.1. Mu Opiate Receptor (OPRM1)

Human mu opiate receptor (OPRM1; GenBank NC_000006.10) is an important component of the self-rewarding system. This receptor is encoded by the OPRM1 gene which is located at the chromosome 6q24-q25. OPRM1 interacts with multiple endogenous opioid peptides including beta-endorphin (Selly and Bidlack, 1992) and endomorphins (Zadina *et al*, 1997). It also mediates the effects of several important opioid analgesic agents and drugs such as morphine, methadone, fentanyl (Pasternak, 1993) and especially heroin (Kreek, 1996).

It depresses cAMP levels, opens G protein-modulated K^+ channel and a third spliced variant act as a morphine-6-beta-glucoronide receptor. Rapid activation of the OPRM1 by heroin and/or its analogs results in euphoric effect that may have conferred reinforcing or rewarding effects of the drug, and thus contributed to the development of drug addiction (Koob, 1992; Wise, 1996).

Almost more than 500 single nucleotide polymorphisms (SNPs) have been identified in OPRM1 (Weizmann Institute of Science, 2007). The SNPs can be found in the promoter region, in intron as well as in exon. Some of the SNPs may have implications to normal physiology, therapeutic, and vulnerability to develop or protect from diverse disease including addictive disease (Bond *et al*, 1998).

2.2. Genotype – Phenotype Correlation

The most prevalent SNPs is 118 A/G in exon 1 which changes amino acid Asn to Asp at position 40. SNP 118 A/G affects beta-endorphin binding affinity of OPRM1 and changes the agonist potency of beta-endorphin for activating the OPRM1 (Bond *et al*, 1998). Furthermore, SNP 118 A/G is associated with nicotine reinforcement in woman (Ray *et al*, 2006). SNPs 118 A/G is also implicated in predisposition to drug addiction. This SNP decreased miotic potency of morphine and active metabolite of morphine; morphine-6-glucoronide (M6G). In addiction, it increased demands of M6G to produce analgesia.

Mutant allele of 17 C/T in exon 1 is associated with drug addiction (Bond *et al*, 1998). The combination of both mutant alleles of 118 A/G and IVS2+691 C/G (intron 2) caused opioid dependence (Szeto *et al*, 2001). However, combination of both mutant alleles of 118 A/G and IVS2+31 G/A caused individual taking heroin consumed higher doses than individual who did not carry this polymorphism (Shi *et al*, 2002). Meanwhile, mutant allele of SNP 802 T/C has shown to produce altered receptor desensitization and receptor signaling with decreased G-protein coupling (Koch *et al*, 2000).

2.3. Method Available for the Detection of OPRM1 Polymorphism

Several methods are currently available for the detection of OPRM1 polymorphism. These include PCR-restriction fragment length polymorphism (PCR-RFLP) (Gelernter *et al*, 1999: Zhang *et al*, 2006), fluorescence resonance energy transfer (FRET)-PCR (Grösch *et al*, 2001), DNA sequencing (Ide *et al*, 2004), SYBR Green method (Zhang *et al*, 2005), and fluorogenic 5' nuclease assay (TaqMan) (Zhang *et al*, 2006; Carlos *et al*, 2007). FRET-PCR, SYBR Green, and TaqMan are the methods in real-time PCR. Each of these methods has its own limitation.

RFLP method used restriction enzyme to digest DNA sample which produce different fragments in different lengths depending on the presence or absence of restriction site in the DNA sequence (Butler, 1998). SNPs that are present on a DNA sequence will be recognized by a restriction enzyme which caused cleaving of the DNA into different lengths of restriction fragments. Thus, the DNA of individuals to be compared may be cleaved by one or several restriction enzyme (Tagu, 2006). As a result, two individuals can present different restriction profiles.

Unfortunately, RFLP methods have some limitations. First, several micrograms of DNA are needed for analysis (Tait, 1997). In addition, initial information about restriction site in the gene need to be recognized. Otherwise, the restriction enzyme will cleave at unwanted nucleotide causing unwanted fragments. Furthermore, different SNPs require different restriction enzyme to be cleaved. Thus, RFLP is not suitable for multiple SNPs as only two SNPs were reported to be used (Gelernter *et al*, 1999: Zhang *et al*, 2006).

Real time PCR is a method to measure the quantity of PCR product in the early phase of PCR reaction (Hunt, 2007). There are two flavors of real-time PCR; probe based and intercalator based. Probe based real-time PCR also known as 5' nuclease assay. This assay requires a pair of PCR primers and additional fluorogenic probe with both a reporter fluorescent dye and a quencher dye attached. Intercalator based method also known as SYBR Green method require a double stranded DNA dye which binds to newly synthesized double stranded DNA and gives fluorescences.

Though real-time PCR provides distinct advantage over traditional PCR, this method has some limitation too. This method is expensive in term of reagents and equipment. Most researchers used real-time PCR to detect 118 A/G (Grösch *et al*, 2001; Zhang *et al*, 2005; Janicki *et al*, 2006; Ray *et al*, 2006; and Hernandez-Avila *et al*, 2007). Rarely researchers used this method to detect others SNPs (Zhang *et al*, 2005 and Hernandez-Avila *et al*, 2007). Hence, real-time PCR is unsuitable for detection multiple SNPs in one reaction.

On the other hand, DNA sequencing is a method to determine the actual nucleotide sequence of the whole region of interest. This method is usually used for validation purpose only. But, some researchers used DNA sequencing to find possible polymorphism in OPRM1 (Ide *et al*, 2004). However, DNA being sequenced must be present at a high enough concentration before sequencing can be performed. Therefore sequencing method is more accurate but it is not suitable for population study.

2.4. Nested Allele-Specific Multiplex Polymerase Chain Reaction

A simple and rapid method is more suitable to be developed to detect polymorphism of OPRM1. The method is nested allele-specific multiplex polymerase chain reaction. This PCR method is a combination of three type of PCR technique; nested, allele-specific and multiplex. Each technique has different advantages that allow more SNPs to be detected.

Nested PCR provides a tool for increasing sensitivity that allowed to 'fish out' the specific amplification product from the 'sea' of non-specific products. It generally uses two primers that are internal to the product of the first PCR. The PCR product from the first PCR is used as template DNA for a second round of PCR with the internal primers. This should yield a smaller PCR product compared with the original product.

Allele-specific PCR is a selective PCR amplification to identify known single base differences in target DNA (SNPs). One of the primers will precisely match one allelic variant of the target sequence at 3' end but it is mismatched to the other. Allele discrimination relies exclusively on the 3' end base of the primer. Thus, successful amplification with SNP-specific primer signals presence of the specific SNP in a sequence.

Meanwhile, multiplex PCR can save the time to detect many SNPs. Different PCR products in varying sizes will be produced and visualized by gel electrophoresis. Multiplex PCR is a variant of PCR which enabling simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers.

In this research a two-step PCR reaction was designed to detect twelve single nucleotide polymorphisms (SNP) in the receptor gene. Those SNPS were located in the exon 1, 2, 3 and intron 2 region. In the first PCR, four pairs of primers is used to amplify exon 1, 2, 3, and intron 2 of OPRM1. Then, the product is used in second PCR with a set of primers that have specific 3'-end that will match or mismatch the single nucleotide change at the specific base. The second PCR apply the allele-specific PCR technique.

CHAPTER 3

OBJECTIVE OF THE STUDY

Objectives of this study are:

1) To develop a simple and rapid PCR method for detecting polymorphism of mu opiate receptor (OPRM1).

2) To validate the PCR method developed.

CHAPTER 4

MATERIALS AND METHODS

4.1. Detection of Genetic Polymorphism Using Polymerase Chain Reaction

This part describes a simple PCR method developed for the simultaneous detection of SNP found on the mu opiate receptor gene, shown in Table 1. A two-step PCR method was used to detect all the SNPs.

Variant name	Gene location
17 C/T	Exon 1
24 G/A	
118 A/G	
440 C/G	Exon 2
454 A/G	
IVS2 +31 G/A	Intron 2
IVS2 +691 C/G	
779 G/A	Exon 3
794 G/A	
802 T/C	
820 G/A	
942 G/A	

Table 4.1: SNPs in mu opiate receptor gene.

4.2. Chemicals, Reagents, and Instruments

Table 4.2 lists the chemicals and reagents and Table 4.3 lists the instrument used in the PCR for the specified alleles of mu opiate receptor gene.

	Chemicals and Reagents	Supplier
1	Agarose LE, Analytical Grade	Promega, Promega Corporation, Madison, USA
2	Biotools DNA Polymerase (recombinant *E. Coli*), 1 U/μL a) Storage buffer: [10mM Tris HCl (pH 8.0), 50 mM KCl, 1 mM EDTA, 0.1% Triton X-100, 50 % glycerol (v/v)] b) Reaction buffer: [10X concentration: 75 mM Tris HCl (pH 9.0), 50 mM KCl, 20 mM $(NH_4)_2SO_4$] c) 50 mM $MgCl_2$ supplied separately	Biotools, Biotechnology & Medical Laboratories, S. A. Spain
3	Deoxynucleoside Triphosphate set (dATP, dCTP, dGTP, dTTP)	Boehringer Mannheim®, Roche Molecular Biochemicals, GmbH-Germany
4	Ethidium bromide (10mg/ml) Molecular Biology Grade	Promega, Promega Corporation, Madison, USA
5	Ethylenediamine-tetraacetic acid (EDTA) Ultra Pure Grade	Promega, Promega Corporation, Madison, USA
6	Gene ruler 100 bp DNA Ladder	Promega, Promega Corporation, Madison, USA
7	Tris base Ultra Pure Grade	Promega, Promega Corporation, Madison, USA
8	Ultra Pure Water (Ω18.2)	Mili-Q® Reagent water system, Milipore MA, USA

Table 4.2: Chemicals and reagents used for PCR genotyping

	Chemicals and Reagents	Supplier
1	Electrophoresis Tank Mini and Maxicell®	Primo Submarine Gel System, ThermoQuest, E-C Apparatus Division, Holbrook, New York
2	Electrophoresis Power Supply EC 250-90	ThermoQuest, E-C Apparatus Division, Holbrook, New York
3	Microcentrifuge	Eppendorf, Hamburg, Germany
4	pH meter	EcoScan, Eutech Instrument, Singapore
5	Autoclave	Kubota, Fujioka, Japan
6	Temperature controlled water bath	Memmert. Schwabach, Germany
7	Thermal cycler	My Cycler Thermal Cycler Biorad
8	Weighing scale	Sartorius, Goettingen, Germany
9	NanoDrop®ND-1000 Spectrophotometer	

Table 4.3: Instruments used for PCR genotyping

4.3. Isolation of DNA

4.3.1. DNA Extraction

The peripheral blood was drawn from the volunteers involved. A kit was used to extract the DNA which was QIAamp DNA mini kit. Then, DNA was purified by using Spin Protocol. This protocol was used to purify of total DNA from whole blood, plasma, serum, buffy coat, lymphocytes, and body fluids using a microcentrifuge (Qiagen, 2007).

First, 20 µl proteinase K was added into the bottom of 1.5 ml microcentrifuge tube. Then, 200 µl blood was added to the tube. After that, 200 µl Buffer AL was added to the tube. All the reagents and sample were mixed by using pulse vortex for 15 seconds. Then, the tube was incubated in water bath at 56°C for 10 minutes. The tube then was briefly centrifuged to remove drops from the inside of the lid.

After centrifuge, 200 µl ethanol (96-100%) was added to the sample and mixed again by using pulse-vortexing for 15 seconds. After mixing, the tube was briefly centrifuged to remove drops from the inside of the lid. Then, mixture from the tube was transferred to the QIAamp mini spin column carefully without wetting the rim. The cap was closed and centrifuged at 8000 rpm for 1 minute. Then, the QIAamp mini spin column was placed in a clean 2 ml collection tube and the tube containing the filtrate was discarded.

Carefully, the cap of QIAamp mini spin column was opened and added with 500 µl Buffer AW1 without wetting the rim. Then, the cap was closed and the column centrifuged at 8000 rpm for 1 minute. Then, the QIAamp mini spin column was placed in a clean 2 ml collection tube and the tube containing the filtrate was discarded.

Then, previous step was repeated but the reagent used was 500 µl Buffer AW2 and centrifuged the tube at 14000 rpm for 3 minute. To eliminate the chance of possible Buffer AW2 carryover, the spin column was placed in a clean 2 ml collection tube and centrifuged at 14000 rpm for 1 minute. The filtrate was discarded. Next, the spin column was placed in 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded.

The cap of spin column was opened and 200 µl Buffer AE was added into the tube. Then, the tube was incubated at room temperature for 1 minute. The reconstituted DNA was stored at -20°C until analysis. The DNA yield was approximately 30 ng/µl. After that, DNA was determined for DNA purity, concentration, and integrity.

4.3.2. Spectrophotometric Determination of DNA Concentration and Purity

DNA concentration and purity was determined by using NanoDrop®ND-1000 Spectrophotometer. This instrument is a full spectrum (220-750nm) spectrophotometer that measures 1 µl of samples with high accuracy and reproducibility. In addition, this instrument is capable of measuring highly concentrated samples without dilution (75X higher concentration than the samples measured by a standard cuvette spectrophotometer).

DNA concentration can be estimated by measuring the absorbance (A) at 260 nm. DNA purity can be estimated from the A_{260}/A_{280} ratio. A pure sample of DNA has the A_{260}/A_{280} ratio at 1.8 and is relatively free from protein contamination. A DNA preparation that is contaminated with protein will have a A_{260}/A_{280} ratio lower than 1.8 (Leninger, 1975).

4.3.3. Determination of DNA Integrity

DNA integrity was determined by using gel electrophoresis. The percentage of the agarose gel used is 2 % and the voltage used is 90 V with time 60 minutes.

4.4. PCR-Based Genotyping

PCR was used to detect the following SNPS (in Table 4.1) of the mu opiate receptor gene. Figure 4.1 showed illustration for the site of amplification of PCR1 and PCR2.

4.4.1. Method Development for First and Second PCR

4.4.1.1.Primer Design

All the primers for first and second PCR were designed manually using the OPRM1 nucleotide sequence (OPRM1; GenBank NC_000006.10) (in Table 4.4). Then, the primers designed were aligned using the BLAST program at NCBI to check for their specificity. Primers for the second PCR were carefully designed to have specific 3'-ends that will match or mismatch single nucleotide change at the specific base. The initial annealing temperature was determined using the formula "T_m=[2(A+T)+4(G+C)]".

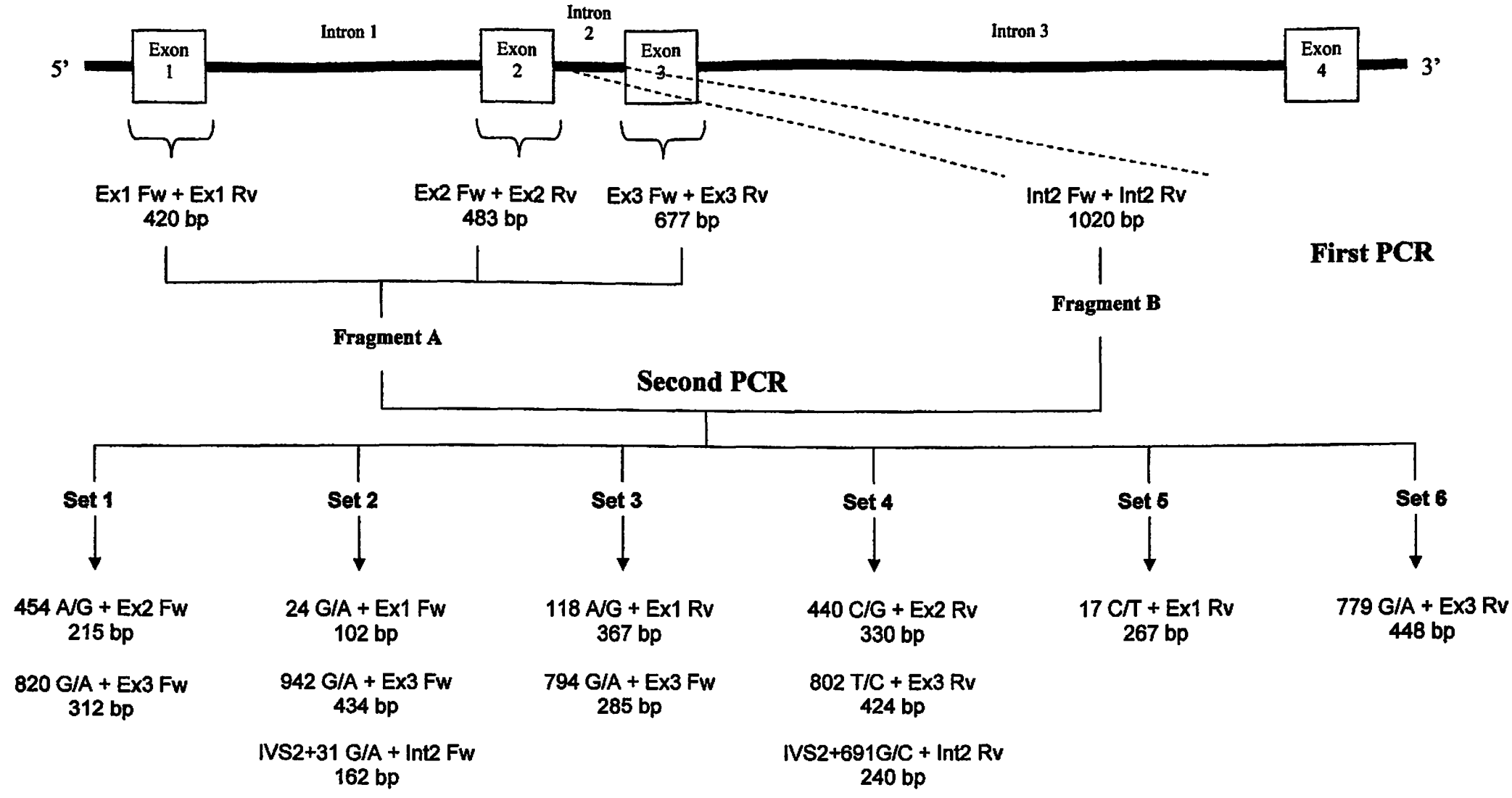

Figure 4.1: Illustration for the site of amplification of first PCR product and second PCR product of OPRM1.

No.	Primer name	Primer sequence from 5' to 3'
1	Ex1 Fw	AAA GTC TCG GTG CTC CTG GCT
2	Ex1 Rv	TGG GAG TTA GGT GTC TCT TTG TA
3	17C Fw	TAC CAT GGA CAG CAG CGC TGC
4	17T Fw	TAC CAT GGA CAG CAG CGC TGT
5	24G Rv	ATG AGT GCA ATT GCT GGC GTT C
6	24A Rv	ATG AGT GCA ATT GCT GGC GTT T
7	118A Fw	CAA CTT GTC CCA CTT AGA TGG CA
8	118G Fw	CAA CTT GTC CCA CTT AGA TGG CG
9	Exn2 Fw	TTC TCA CTC TTC TTC CTT TAT CTC
10	Exn2 Rv	GAC TAA GAC AAT GGG GCT CTC CA
11	440C Fw	CCA TCC TTT GCA AGA TAG TGA TCT C
12	440G Fw	CCA TCC TTT GCA AGA TAG TGA TCT G
13	454A Rv	GGT GAA TAT GCT GGT GAA CAT GTT
14	454G Rv	GGT GAA TAT GCT GGT GAA CAT GTC
15	Int 2 Fw	TAG ATT TCC GTA CTC CCC GAA
16	Int 2 Rv	CGC AAG ATC ATC AGT CCA TAG
17	IVS2 +31G Rv	AAC ATA TCA GGC TGT GAA CCC
18	IVS2 +31A Rv	AAC ATA TCA GGC TGT GAA CCT
19	IVS2 +691G Fw	GCT CTG GTC AAG GCT AAA AAT G
20	IVS2 +691C Fw	GCT CTG GTC AAG GCT AAA AAT C
21	Exn3 Fw	GCC TTA AGT TAG CTC TGG TCA
22	Exn3 Rv	TGT CAT CCC CAG TAG ATA TAC C
23	779G Fw	GCT ATG GAC TGA TGA TCT TGC G
24	779A Fw	GCT ATG GAC TGA TGA TCT TGC A
25	794G Rv	CTT TGG AGC CAG AGA GCA TGC
26	794A Rv	CTT TGG AGC CAG AGA GCA TGT
27	802T Fw	TCA AGA GTG TCC GCA TGC TCT
28	802C Fw	TCA AGA GTG TCC GCA TGC TCC
29	820G Rv	GAT CCT TCG AAG ATT CCT GTC
30	820A Rv	GAT CCT TCG AAG ATT CCT GTT
31	942G Rv	TGC CAA GAA ACA GTC TGG AAC
32	942A Rv	TGC CAA GAA ACA GTC TGG AAT

Table 4.4: List of primers of mu opiate receptor genotyping

4.4.1.2.Standard PCR Condition

The standard PCR condition is tabulated in Table 4.5. Concentration for each PCR component is adjusted until optimized concentration is obtained. Similarly, number of cycles, temperature and time used also adjusted to obtain optimum sensitivity and specificity. PCR gradient mode is applied to obtain optimum annealing temperature by using My Cycler Thermal Cycler Biorad. Gel concentration is set at 2 % with development time 60 min at 90 volt. Gel concentration may change for optimum development and separation.

PCR Component	Concentration
Buffer (10x)	1x
$MgCl_2$ (50 mM)	1.5 mM
dNTP (10 mM)	0.2 mM
Taq Polymerase (1 unit/µl)	1 µl
Primer (5 pmol)	0.25 pmol
DNA (~50 ng)	4 ng

Cycle Condition	Guideline
Denaturation	Temperature: 95 °C
	Time: 5 min on initial cycle; 30 sec to 1 min on rest
Annealing	Temperature: 5 °C below T_m of primers; not lower than 40 °C.
	Time: 30 – 45 sec. This is the step where you would use a gradient.
Extension	Temperature: 72 °C
	Time: ~1 min/kb of expected product; 5 – 10 min on last cycle.
Number of cycles	~ 30 cycles

Table 4.5: Standard PCR condition

4.5. Reconstitution of Primers

Once the primers were received, they need to be reconstitution first before it can be used. The primers were first spun at 14 000 g for 1 min. This will make the lyophilized powder to be collected at the bottom of the tube. The OD of the primers was provided in product information sheet. The amount of Mili-Q water needed was calculated by using formula below.

$$\text{pmol} = \frac{\text{number of OD x 33 x } 10^6 \text{ pg}}{\text{number of mers for the primer x 330}}$$

$$\text{Amount of water (µl)} = \frac{\text{total pmol calculated above (pmol)}}{\text{desired stock concentration of primer (pmol/µl)}}$$

For example, the primer Y has 5 OD and 20 mers.

So, amount of water (µl)

$$pmol = \frac{5 \times 33 \times 10^6}{20 \times 330}$$

$$pmol = 25000$$

$$\therefore water = \frac{25000 pmol}{100 pmol / \mu l}$$

$$water = 250 \mu l$$

Working stock, then, prepared at a concentration of 5 pmol/µl.

Half of the calculated amount of water was first added. Then, the tube was shaken thoroughly or vortex mixed briefly to dissolve the primers. The remnant water was added and vortex briefly again. It was then left overnight at 4 °C before it was aliquoted to be kept at -20°C.

4.6. Calculation of Working Solution

The quantity of stock reagents used was calculated for a total PCR volume of 25 µl using the formula below:

$$M_1V_1 = M_2V_2$$

(initial stock concentration) x (volume needed) = (final concentration) x (volume sample)

For instance, $MgCl_2$ were stored at 50 mM, but the working concentration used was 2 mM, thus:

$$volume = \frac{(25 \times 2)}{50}$$

$$volume = 1\mu l$$

4.7. PCR-Genotyping (Optimized Method)

4.7.1. First PCR

A two-step PCR method was used to detect all the SNPs. In the first PCR reaction, four parallel reactions were carried out in two sets of different tubes. The first set contained three pairs of primers that were used to amplify fragment A. First pairs of primer were primer Ex1 Fw and Ex1 Rv that were used to amplify exon 1 of the OPRM1 gene. The second pairs of primers were Ex2 Fw and Ex2 Rv. Those primers were used to amplify exon 2 of the OPRM1 gene. Primer Ex3 Fw and Ex3 Rv were used to amplify exon 3 of OPRM1 gene. The second set contained primer Int2 Fw and Int2 Rv were used to amplify fragment B which is intron 2 of OPRM1 gene.

This PCR product was subsequently used as a template in the second allele-specific PCR reaction. Each reaction mixture was divided into two fractions. The first fraction contained approximately 0.25 pmol of each primer, 50 ng genomic DNA, and 10.5 µl Mili-Q water. All the reactions were carried out using a My Cycler Thermal Cycler Biorad. The second fraction contained 1X Reaction buffer, 1.5 mM $MgCl_2$, 0.5 mM dNTP mixture, 1.0 U of *Taq polymerase* and 5.25 µl Mili Q-water. Approximately 10 µl of second fraction was added to the first reaction to make up the final to 25 µl.

Amplification was performed according to the following procedures; pre-denaturation at 95 °C for 1 min, denaturation at 95 °C for 30 sec, annealing at 53.5 °C for 30 sec and extension at 72 °C for 30 sec, followed by a final extension at 72 °C for 1 min after 30 cycles. Eight microliters of the first PCR product was analyzed on 3 % agarose gel and 1X TBE buffer. The first PCR products were diluted 1 in 50 with autoclaved Mili-Q water and then were used for the second step PCR for single base changes identification.

4.7.2. Second PCR for 24 G/A, 440 C/G, 802 T/C, 942 G/A, IVS +31 G/A and IVS +691 G/C

Six parallel allele-specific PCR were carried out in two sets of two tubes. First set was applied to detect SNPs 24 G/A, 942 G/A, and IVS +31 G/A while the second set were used to detect SNPs 440 C/G, 802 T/C, and IVS +691 G/C. Each tube contained 2 µl PCR product from the first PCR that contained fragments A and B (diluted 50X), 1X Reaction buffer and 0.2 mM dNTP.

The first set contained 2.0 mM $MgCl_2$, 1.5 U of *Taq polymerase*, 0.15 pmol 942 G/A and Ex3 Fw (common primer), and 0.3 pmol of 24 G/A, Ex1 Fw (common primer), IVS +31 G/A, and Int2 Fw (common primer). The second set enclosed 1.5 mM $MgCl_2$, 1.0 U of *Taq polymerase*, 0.15 pmol 440 C/G, Ex2 Rv (common primer), 0.2 pmol IVS +691 G/C, and Int2 Fw (common primer) and 0.3 pmol of 802 T/C, Ex3 Rv (common primer). Then, Mili-Q water was added to make up the final volume to 25 µl.

For both Set 1 and 2, the PCR reaction consisted of pre-denaturation at 95 °C for 1 min, followed by 30 cycles, each cycle involving denaturation at 95 °C for 30 sec, annealing at 64.4 °C (set 1) and 65 °C (set 2) for 30 sec and extension at 72 °C for 30 sec and final extension at 72 °C for 1 min. Eight microliters of the PCR product was analyzed on 3 % agarose gel and 1X TBE buffer.

4.8. Validation

For validation of PCR method, product of first PCR was sent for sequencing.

4.9. Gel Electrophoresis

4.9.1. Preparation of Agarose Gel

After, PCR completed, the product was separated by using agarose gel electrophoresis. The required of agarose powder was weighed and dissolved in 100 ml of 1x TBE buffer by heating the slurry in a microwave oven. The amount of agarose powder depends on the percentage of agarose gel required.

After heating, 5 µl ethidium bromide was added to the slurry and mixed thoroughly. Then, the warm agarose was poured into a mold. Precaution should be taken during added ethidium bromide and during pouring the warm agarose due to carcinogenic properties present in the ethidium bromide. Do not inhale the vapour during this time.

Air bubbles under or between the teeth of the comb were removed. If bubbles present the separation may be interfered. After the gel was hardened, the comb was removed carefully and the gel was mounted in the electrophoresis tank. Sufficient amount of 1x TBE buffer was added until the gel was sinking.

Eight microliter of DNA samples were mixed with 2 µl of 6x gel-loading dye and loaded carefully into the slots of submerged gel. A voltage of about 1-5 volt/cm is applied (distance is measured from negative to positive electrode). Then, the power was checked to ensure the leads were properly attached. Once, they were properly attached many bubbles were generated at both positive and negative electrode. After the samples have run sufficiently, the electric current is switched off and the leads were removed from the tank.

4.9.2. Gel Image Capturing

Then, the gel is taken out from the tank and was placed onto an ultraviolet transluminator. It was examined under UV light and its image was previewed using the Image Capture System. The size and resolution of the image was adjusted before a clear picture of the gel was captured for future analysis.