CHAPTER 2

RESEARCH DESIGN AND METHODS

2.1 An overview

The research design was divided into 2 sections, the first section was subject selection and the second was to determine the genetic polymorphisms.

The study population was composed of 90 Thai subjects which were divided into 3 groups, two were moderate and severe alcohol dependence groups and non-alcohol dependence group as a control. Sixty alcohol-dependent patients were recruited from the psychiatric OPD of Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand between April to December, 2009. Thirty non-alcohol dependences from faculty of medicine, Chiang mai University were recruited as controls.

Subjects were asked to fill in the self-reported questionaires and interviewed face-to-face by the researcher. All were asked to complete the questionnaires containing three parts. The first part was about their general information: demographic characteristics, city residence, occupation, medical and family history. Then subjects were assessed of drinking status using MINI (Mini International Neuropsychiatric Interview) Thai version 5.0.0 which is the second part of the questionnaires. In the third part, the alcohol dependence patients were assessed for severity of dependence using SADQ (Severity of Alcohol Dependence Questionaires). The patients who had a score lower than 30 were defined as moderate alcohol dependence while patients with the scores over 30 were defined as severe dependence. The summary of the research design is shown in Figure 3.

Buccal cells were collected after the subjects were interviewed with the questionaires. Buccal cells were used as a source of DNA which later were typed at a SNP of dopamine receptor D2 gene by PCR-RFLP technique. All data records were kept confidentially by coding all subjects throughout the study. Finally, the data were analyzed statistically using Chi-square test and spearman correlation. The summary diagram for polymorphism detection and DNA genotyping is summarized in Figure 4. The study protocols, questionnaires and consent forms were already approved by the Institutional Ethics Committee of the Faculty of Medicine, Chiang Mai University, Thailand.

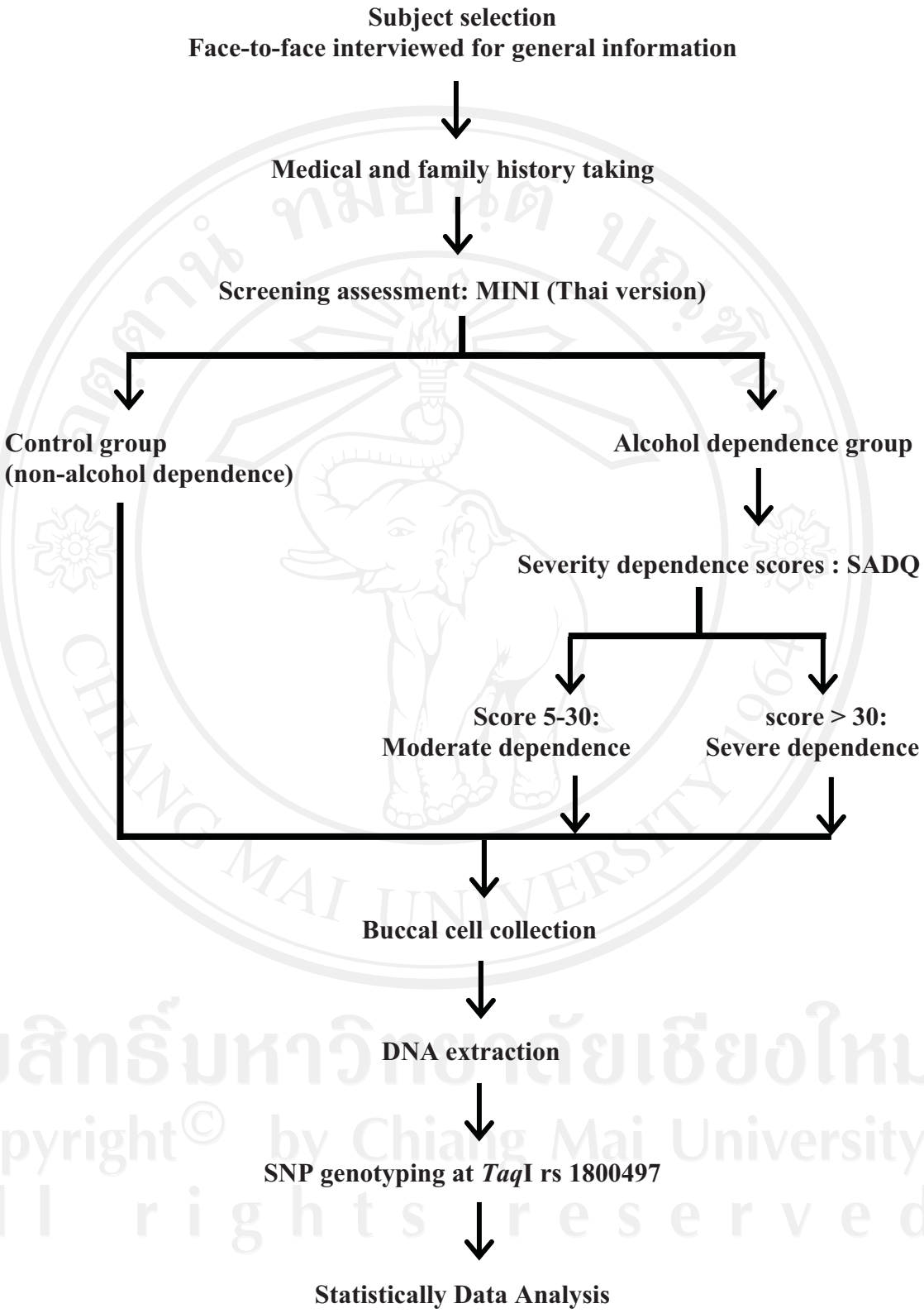


Figure 3 Summary of research design

2.2 Method

2.2.1 Population calculation and screening assessment

The population size was calculated from the formulation below

Sample size = $$\frac{(Z_\alpha\sqrt{2\overline{PQ}} + Z_\beta\sqrt{PcQc + PtQt})^2}{(Pc - Pt)^2}$$

Z_α = The Z value corresponding to the type I error

Z_β = The Z value corresponding to the type II error

Pc = Current estimate of population proportion P1 (Non-Exposed or Control Group)

Pt = Current estimate of population proportion P2 (Exposed or Treated Group)

Qc = 1- *Pc*

Qt = 1- *Pt*

$\overline{P} = \frac{Pc + \lambda Pt}{1 + \lambda}$ when $\lambda = \frac{nt}{nc}$ in equals sample sizes nt = nc, $\lambda = 1$

$\overline{Q}$ = 1 - $\overline{P}$

The population size calculation was based on the study of Blum et al., 1991. The optimal number of subject from calculation is more than 22 persons in both alcoholic and control groups. After signing the informed consent, all subjects were face-to-face interviewed about their general information, medical and drinking history. Alcohol dependence were assessed for drinking status using MINI Thai version 5.0.0 (Amorim *et al.,* 1998; Lecrubier *et al.,* 1997; Sheehan *et al.,*1997;

Sheehan *et al.,* 1998; Kittirattanapaiboon and Khamwongpin, 2005) as the screening questionnaires. The study population were defined into 3 groups (30 persons each) as mild to moderate dependence, severe dependence and controls (non-alcohol dependence): males or females with age and sex matched in all groups, age ranging from 20-70 years old. The criteria for three study groups were shown below

Group 1 and 2 are the alcohol dependence patients from the alcohol detoxification unit, Department of Psychiatry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. The members of alcohol dependence groups are subjects who answered "yes" at least three of seven questions in Module J2 with the exclusion criteria:

1. Related with other subjects in this study
2. Current polysubstance dependence with the exception of nicotine use or occasional at most once per month marijuana use
3. Physical or mental obstruction to complete the interview such as severe alcohol-related medical complication, being sedated by benzodiazepine or another medication, dementia, delirium, mood disorder, psychosis (as schizophrenia) or Central Nervous System impairment
4. Being in acute alcohol withdrawal status

(Kono *et al.,* 1997; Lawford *et al.,* 1997; Connor *et al.,* 2008)

The severity of alcohol dependence was scored using the SADQ. The patients in group 1 were defined as mild to moderate alcohol dependence with a score lower than 30 and subjects in group 2 were defined as severe dependence patients with a score over 30.

The subjects in the control group are whom answered “no” both in module J1 Alcohol abuse and dependence and K1 Non-alcohol psychoactive substance use disorders in MINI questionnaires with the exclusion criteria below

1. related with other subjects in this study

2. Current polysubstance dependence with the exception of nicotine use or occasional at most once per month marijuana use

3. Physical or mental obstruction to complete the interview such as severe alcohol-related medical complication, being sedated by benzodiazepine or another medication, dementia, delirium, mood disorder, psychosis (as schizophrenia) or Central Nervous System impairment (Kono *et al.,* 1997, Lawford *et al.,* 1997, Noble *et al.,* 1998b)

2.2.2 Buccal cell collection

The collection procedure was modified from the standard operating procedure for forensic DNA analysis method of department of Forensic Medicine, Faculty of Medicine, Chiang mai University. Buccal cells were collected from the subjects by scraping 10 times using the blunt end of soft toothpicks. The sample was soaked in 1ml of distilled water in 1.5ml microcentrifuge tube and frozen at -20 ^{o}c until DNA extraction.

2.2.3 DNA extraction from buccal cells

The buccal cells samples were centrifuged at 14,000 rpm for 1 minute then the supernatant was removed. The pellet was washed in 1ml of distilled water for 3 times before 295 µl of distilled water, 5 µl of proteinase K solution (10mg/ml) and chelex resin were added. The sample tubes were incubated at 55 ^{o}c for 1 hour then, boiled

for 8 minutes. The extract was stored at 4 ^{o}c until used as a template for DNA amplification.

2.2.4 DNA Amplification

The method was modified from Grandy *et al.,* 1993 for DNA amplification of 310 bp product with some modification of primers sequences as shown in Table 3.

Table 3 Primer sets for the amplification of the *TaqI* rs1800497

Primers use	Sequences
Primers set 1	Forward: 5’ GCT CAC TGC AAC CTC TGC CTC CTG G 3’ Reverse: 5’ ACT GGA CTC CCC TGC ACC TAG CTG C 3’
Primers set 2	Forward: 5’ ACG GCT GGC CAA GTT GTC TA 3’ Reverse: 5’ CCT TCC TGA GTG TCA TCA AC 3’
Primers in Grandy *et al.,* 1993	Forward: 5’ CCG TCG ACG GCT GGC CAA GTT GTC TA 3’ Reverse: 5’ CCG TCG ACC CTT CCT GAG TGT CAT CA 3’

Primer set 1

CGGCTCACTG CAACCTCTGC CTCCTGGGTT CAAGGAATTC TCCTGCCTCA GCCTCCCTGG
TAGTTGGGAT TACAGGCACG TGCCACCATA CCCAGCTAAA TTTTGTATTT TTAGCAGAGA
CAGGGTTTTG CCATGTTGGC CAGGCTGGCC TCAAACTCTT GATATCAGGT GATCTGCCTG
CCTCAGCCTC CCAAAGTGCT GGGATTACAG ACGTGAGCCA CCACGGCTGG CCAAGTTGTC
TAAATTTCCA TCTCGGCTCC TGGCTTAGAA CCACCCAGAG TGGCCACTGA CGGCTCCTTG
CCCTCTAGGA AGGACATGAT GCCCTGCTTT CGGCTGCGGA GGGCCAGTTG CAGGGGTGTG
CAGCTCACTC CATCCTGGAC GTCCAGCTGG GCGCCTGCCT
Y
GACCAGCACT TTGAGGATGG CTGTGTTGCC CTTGAGGGCG GCCAGGTGGG CGGGTGTCCA
GCCCACCTTG TTGCGGGCGT GGACATTTGC GTGATGTTCT AGGAGGTTGA TGACACTCAG
GAAGGTGCTC CTCTGGACCG CCAGGTGGAG GGGTGTCCAG CCTGACTGCT CTGCAGCATT
GGGGTCAGCC CCACACTGCA GCAGTGCTGA CACCACCGCC TCCTCCCCGT GGCGTGCAGC
TAGGTGCAGG GGAGTCCAGT TCACAGCTCC AAGAGCACCC ATGTTTGCGT GGCTCTCTGC
CAGCAGATGG ATGATCTCCA GGTGGCCCTT GTAGGCTGCT AGATGCAGGG GTGTCCAGCC
CTGGTGGGTG GGCAGCTCAA GGCTGGCTCC GTACCTGAGC

Forward primer binding site:
5’ GCT CAC TGC AAC CTC TGC CTC CTG G 3’ length 25 bp

Reverse primer binding site:
5’ ACT GGA CTC CCC TGC ACC TAG CTG C 3’ length 25 bp

Y represent the C/T SNP position

Comparison of primers set 2 with Grandy *et al.*, 1993 primer

CGGCTCACTG CAACCTCTGC CTCCTGGGTT CAAGGAATTC TCCTGCCTCA GCCTCCCTGG

TAGTTGGGAT TACAGGCACG TGCCACCATA CCCAGCTAAA TTTTGTATTT TTAGCAGAGA

CAGGGTTTTG CCATGTTGGC CAGGCTGGCC TCAAACTCTT GATATCAGGT GATCTGCCTG

CCTCAGCCTC CCAAAGTGCT GGGATTACAG ACGTGAGCCA CCACGGCTGG CCAAGTTGTC

TAAATTTCCA TCTCGGCTCC TGGCTTAGAA CCACCCAGAG TGGCCACTGA CGGCTCCTTG

CCCTCTAGGA AGGACATGAT GCCCTGCTTT CGGCTGCGGA GGGCCAGTTG CAGGGGTGTG

CAGCTCACTC CATCCTGGAC GTCCAGCTGG GCGCCTGCCT

Y

GACCAGCACT TTGAGGATGG CTGTGTTGCC CTTGAGGGCG GCCAGGTGGG CGGGTGTCCA

GCCCACCTTG TTGCGGGCGT GGACATTTGC GTGATGTTCT AGGAGGTTGA TGACACTCAG

GAAGGTGCTC CTCTGGACCG CCAGGTGGAG GGGTGTCCAG CCTGACTGCT CTGCAGCATT

GGGGTCAGCC CCACACTGCA GCAGTGCTGA CACCACCGCC TCCTCCCCGT GGCGTGCAGC

TAGGTGCAGG GGAGTCCAGT TCACAGCTCC AAGAGCACCC ATGTTTGCGT GGCTCTCTGC

CAGCAGATGG ATGATCTCCA GGTGGCCCTT GTAGGCTGCT AGATGCAGGG GTGTCCAGCC

CTGGTGGGTG GGCAGCTCAA GGCTGGCTCC GTACCTGAGC

Forward primer binding site of primer set 2:
5’ ACG GCT GGC CAA GTT GTC TA 3’ length 20 bp
5’ CCG TCG ACG GCT GGC CAA GTT GTC TA 3’
Forward primer binding site of Grandy *et al.,* 1993

Reverse primer binding site of primer set 2:
5’ C CTT CCT GAG TGT CATCAAC 3’ length 20 bp
5’ CCG TCG ACC CTT CCT GAG TGT CATCA 3’
Reverse primer binding site of Grandy *et al.,* 1993

Y represents the C/T SNP position

2.2.5 Polymorphism detection

2.2.5.1 Polymerase chain reaction

PCR was performed in total volume of 20 µl. The reaction mixture in final volume contained 0.25mM of each dNTPs, 0.5 Unit of Taq DNA polymerase, 0.25µM of each primers, PCR buffer (20mM Tris-HCl pH8.4, 50mM KCl, 1.5mM $MgCl_2$, 0.01% tween 20 and 0.05% BSA) and 2µl of DNA extract was used as a template.

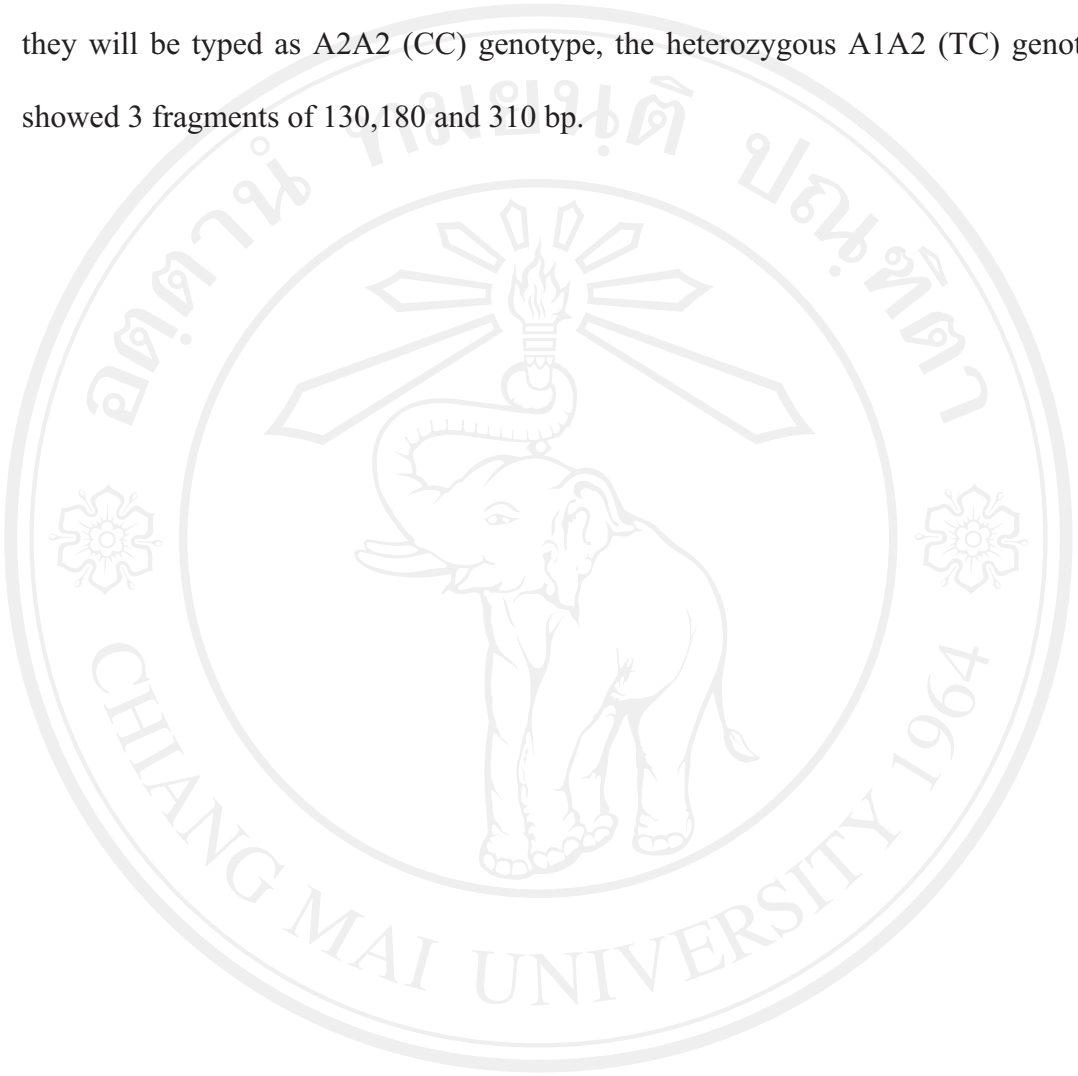
The PCR was carried out in a mastercycler (Eppendorf Germany). Different annealing temperature ranging from 50-57°c was used to optimize the reaction according to the following protocol: initial denaturation at 94 °c for 2 min, then 94°c for 30sec, annealing temperature 30 sec and 72°c for 1min 30 sec for 35 cycles. The PCR products were checked for 310 bp fragment using 2% agarose gel electrophoresis.

2.2.5.2 SNP genotyping

The SNP was analyzed by TaqI restriction enzyme digestion. The recognition site for this enzyme is T!CGA. A mastermix containing 1 U of TaqI (Invitrogen), 1 µl of 10x react 2 buffer and 7µl of distilled water was prepared and added to 1µl of PCR product (about 200ng of DNA) and incubated at 65°C overnight. Paraffin oil was used as an evaporation barrier. A positive control containing the *TaqI* restriction site was included in all experiments to address the problem of false genotyping.

4µl of digested product were mixed with 1µl of loading dye and loaded onto 8.5% polyacrylamide gel in 1xTBE buffer carried out with the BioRad electrophoresis apparatus using 70 volt for 16 hour at room temperature. The DNA fragments were visualized by silver staining. The 3 different genotypes were designated according to

fragment patterns as follow: a single fragment of 310 bp was determined as A1A1 (TT) genotype, if the product was digested into 2 fragments of 130 bp and 180 bp they will be typed as A2A2 (CC) genotype, the heterozygous A1A2 (TC) genotype showed 3 fragments of 130,180 and 310 bp.



Buccal cell collection

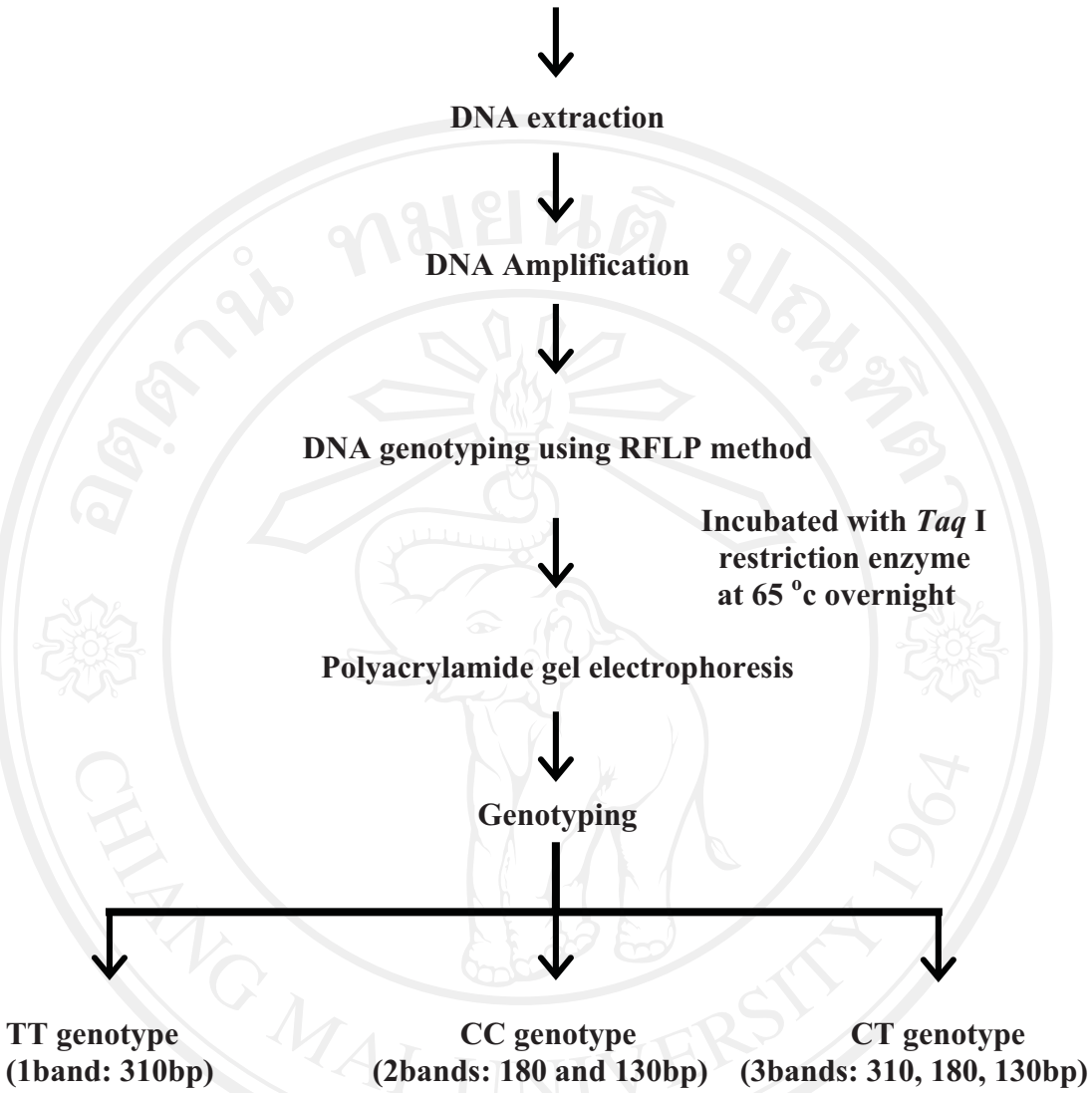


Figure 4 Polymorphism detection and DNA genotyping method

2.3 Statistical analysis

1. Genotypes were investigated and compared the percentage of C/T SNP allele percentage of C/T SNP genotypes, and allele frequency at study site using the formulation of

Percentage calculation:

$$\frac{\text{Number C/T SNP allele or C/T SNP genotype in group}}{\text{Total number of C/T SNP alleles or C/T SNP genotypes in group}} \times 100$$

Allele frequency calculation from genotypes:

Frequency of the A allele:

$$\frac{\text{Total number of A alleles in the population}}{\text{Total number of alleles in population for that locus}}$$

2. The proportion of T allele and C allele were calculated between dependence patients and control group using the formulation of

Proportion of allele calculation:

Proportion of allele in each group:

$$\frac{\text{Frequency of T allele in each group}}{\text{Frequency of C allele in each group}}$$

3. To determine the significance of the difference in the distribution of genotypes and alleles in alcoholic patients and control group were calculated by Pearson Chi-square (χ^2) analysis.

4. To find correlations of percentage of genotypes between dependence patient groups and control group using spearman correlation (r) analysis.

All analyses were conducted with the SPSS Version 15.0 and *p* value of 0.05 was considered to be statistically significant.