CHAPTER II

RESEARCH DESIGN AND METHODS

2.1 Research design

To study the mutations in all coding regions of the *TP63* gene, genomic DNA was extracted from peripheral blood samples taken from affected individuals. Mutation analysis was performed by Polymerase Chain-Reaction (PCR) to amplify specific DNA sequences (Boehm, 1989). This method has been used extensively to analyze *P63* mutations in humans. Then the PCR products were sent to be sequenced by the direct gene sequencing method. The DNA sequences were analyzed by Sequencher 4.8 (Genecodes, Ann Arbor, Mich., USA). All mutations were confirmed by repeat sequencing. The diagram below **(Figure 2.1)** shows the procedures used to investigate the mutations of the *TP63* gene.

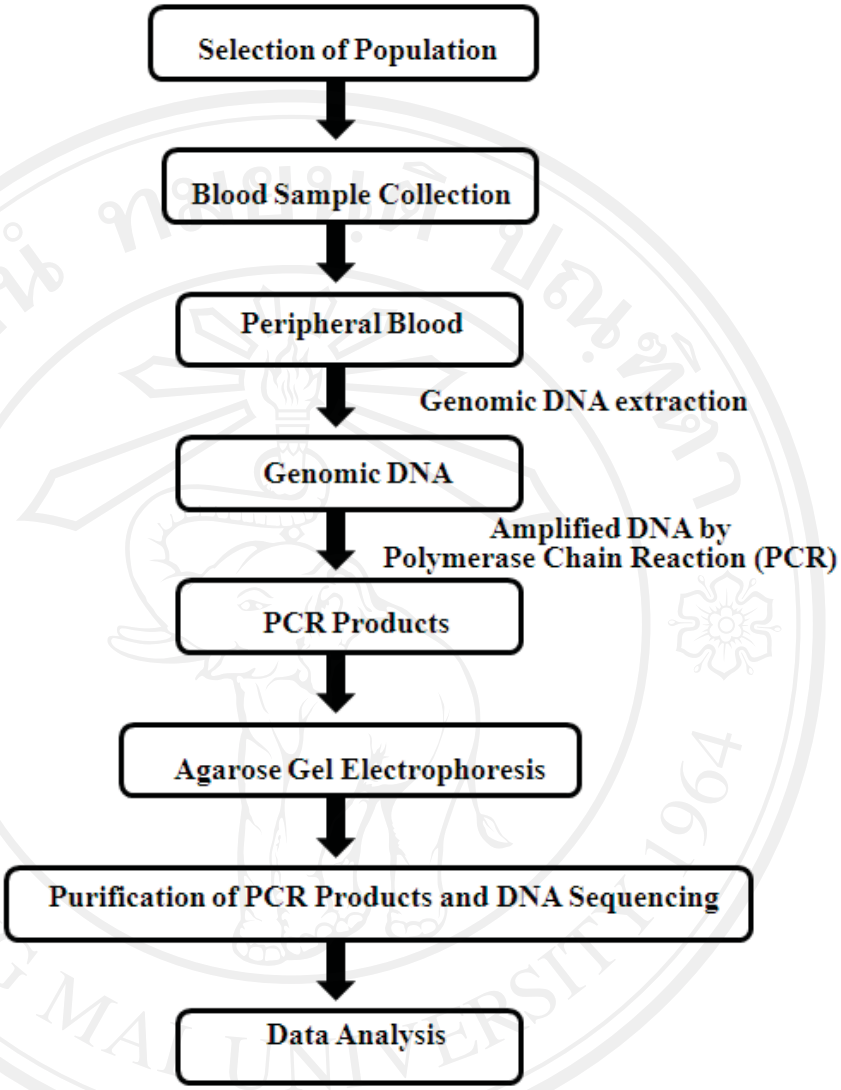

Figure 2.1 Summary of all procedures in this study.

2.2 Methods

2.2.1 Selection of population

Patients: Ten cases of non-syndromic hypodontia and 10 cases of non-syndromic orofacial clefts and 10 cases of syndromic hypodontia and orofacial clefts were recruited from the dental clinics and hospitals in various parts of Thailand. All patients were screened for the presence of hypodontia and orofacial clefts. The inclusion criteria for non-syndromic hypodontia were any congenitally missing teeth and an otherwise unremarkable health history. The inclusion criteria for non-syndromic orofacial clefts were any types of orofacial cleft, such as cleft lip, cleft palate, bifid uvula, or cleft lip with cleft palate, and an otherwise unremarkable health history. The inclusion criteria for syndromic hypodontia with/without orofacial clefts were congenitally missing teeth, with or without any types of orofacial cleft, such as cleft lip, cleft palate or cleft lip with cleft palate, and other remarkable phenotype. The patients received clinical and radiographic examinations. Medical and dental histories were recorded. Informed consent was obtained from all patients, or from their parents if they were under 18 years old. The study was approved by the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University.

The total 30 patients were included in this study, consisting of 10 non-syndromic hypodontia, 10 non-syndromic orofacial clefts, and 10 syndromic hypodontia with/without orofacial clefts **(Table 2.1)**. The Craniofacial Genetics Laboratory (CGL) DNA number and phenotype of all patients in this study were also shown in **Table 2.2**.

Table 2.1 Summary of the relevant data of all patients

Group of patients	**Number**		
Non-syndromic hypodontia	10		
Non-syndromic orofacial clefts	10	Cleft Lip (CL)	1
		Cleft Lip/Palate (CL/P)	9
Syndromic hypodontia with/without orofacial clefts	10		
TOTAL	**30**		

Table 2.2 Craniofacial Genetics Laboratory (CGL) DNA number and phenotype of all patients in this study

Number	(CGL) DNA number	Phenotype
1	001	Hypodontia, Ectodermal Dysplasia: blond hair
2	004	Acrocardiofacial syndrome
3	006	Hypodontia (#18)
4	007	Hypodontia (#38,48)
5	008	Hypodontia (#38)
6	017	Hypodontia, Ectodermal dysplasia: uncombable hair
7	022	Hypodontia (#31,41), Peg-shaped lateral incisors
8	023	Bilateral CL/P and Polydactyly
9	024	Hypodontia (#13,23)
10	029	Hypodontia (#14,15,24,25,34,35,44,45)
11	039	Hypodontia (#13, 22, 23, 31, 41,17)
12	040	Mammary hypoplasia, Eyes anomalies
13	181	EEC syndrome
14	182	EEC syndrome
15	184	Hypodontia, Ectodermal Dysplasia: blond hair
16	210	Hypodontia (#12,22)
17	220	CL/P
18	239	CL/P
19	249	CP and Tongue tie
20	253	CP and Tongue tie
21	289	CL/P
22	294	CL
23	306	CL/P
24	307	CL/P
25	311	CL/P
26	316	CL/P
27	319	CL/P
28	320	CL/P
29	323	Hypodontia (#42)
30	431	Hypodontia (#13,23)

Control samples: All 100 control individuals were of Thai blood donors who had no hypodontia or orofacial clefts upon clinical examination.

2.2.2 Blood sample collection

The blood samples were obtained from the peripheral blood, using standard methods as follows:

1. Five milliliters of blood were drawn from each patient, using lavender-top Vacutainer® EDTA tubes **(**Becton Dickinson**,** Franklin Lakes**,** NJ**,** USA**).**

2. The blood was kept cool, but not frozen, until the blood lysis was performed.

2.2.3 Genomic DNA preparation

The genomic DNA preparation was performed using the standard inorganic salting out method **(Figure 2.2)**. First, the blood was lysed with 10 mL of Red Cell Lysis Buffer (RCLB). The mixture was subsequently centrifuged at 3,000 rpm for five minutes and the supernatant was discarded. The pellet residue was ground again and re-suspended in 10 mL RCLB. After mixing gently with 2 mL of White Cell Lysis Buffer (WCLB), the cells were lysed and the mixtures became viscous. Ten microliters of Proteinase K solution (20 mg/mL) were added to 900 µL of the mixture and incubated in a water bath at 50-56°C for 1.30-2 hours. The digested protein was separated by salting out with 300 µL of 6 M NaCl. To complete protein precipitation, the mixture was centrifuged at 12,000 rpm for five minutes. The clear supernatant was subsequently transferred to new tubes and DNA was precipitated with 600 µL of cool isopropanol. The white, fluffy DNA was then hooked out, centrifuged at 12,000 rpm for five minutes, rinsed with 1,000 µL of 70% ethanol to wash out the excess salt, centrifuged again at 12,000 rpm for five minutes, and kept dry at room temperature

for 1-2 hours. Finally, the DNA was dissolved in 200 µL of 10 mM Tris-base and 1 mM EDTA (TE) buffer and was ready to use for the experiment. The pure DNA was diluted to a 1:10 concentration before the Polymerase Chain-Reaction (PCR) was performed.

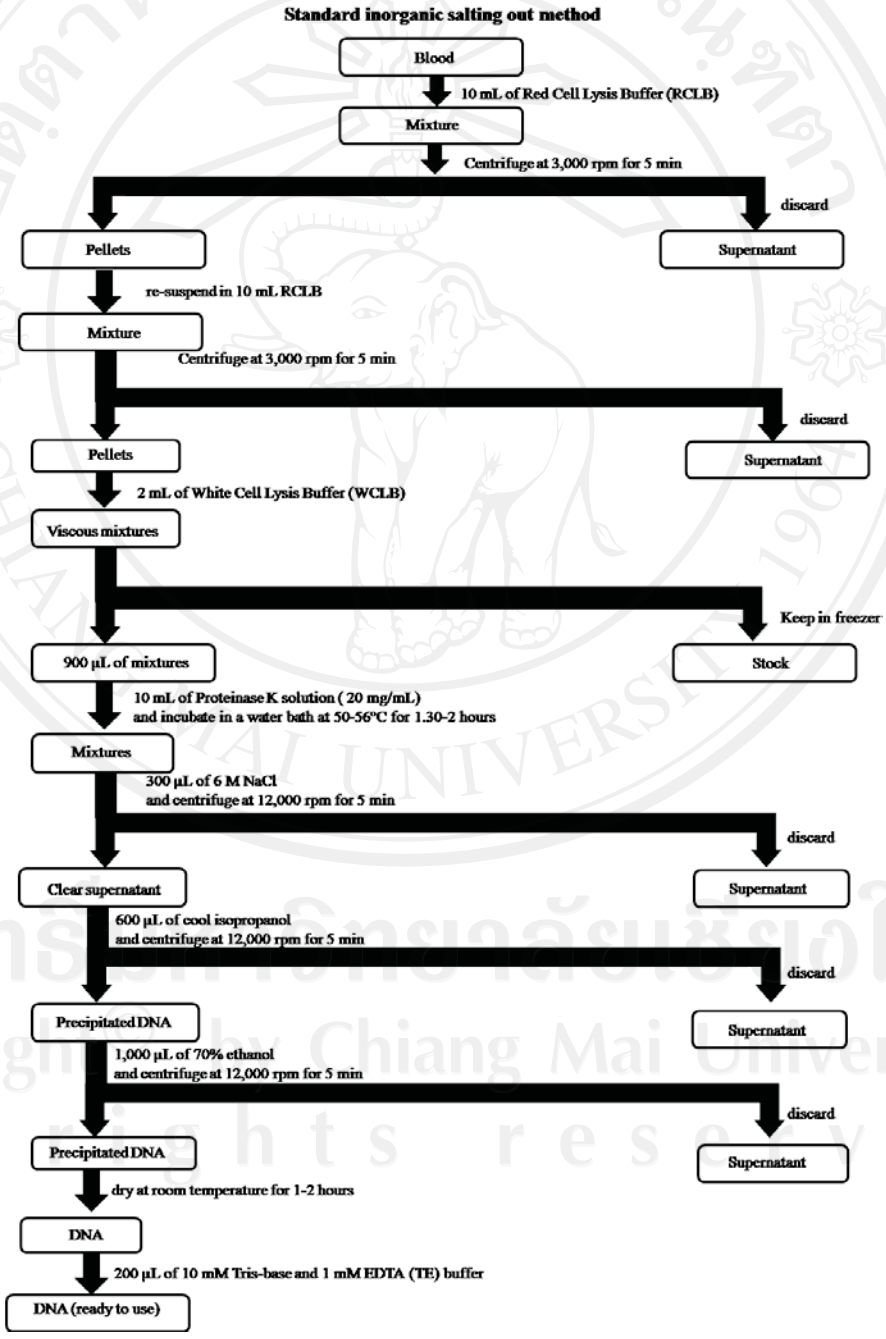

Figure 2.2 Diagram presents the genomic DNA preparation.

2.2.4 *TP63* mutation analysis

Mutation analysis was performed, using a Polymerase Chain Reaction (PCR) using an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) and direct sequencing of 16 exons of the *TP63* gene.

2.2.4.1 Primer designs

The intronal primer sequences were designed to cover the coding regions of *TP63*. The primers that used in this study were described in(Holder-Espinasse et al., 2007; Leoyklang et al., 2006). The primer of exon 14 had two reverse sequences because the results of the first primer for reverse sequence from Leoyklang et al. were not readable (references from Holder-Espinasse et al., 2007; Leoyklang et al., 2006) **(Table 2.3)**.

2.2.4.2 PCR optimization

In this study, various parameters of PCR condition were optimized. The parameters, including the designed primers, the annealing temperatures, and the concentrations of the PCR product components, such as buffer, $MgCl_2$, dNTPs, H_2O, *Taq* Polymerase, DNA, and primers, were optimized to contribute the high quality of PCR products without the non-specific amplified products. The procedures and optimized protocols are as follows:

For PCR amplification of exon 1, 2, 3, 3', 4, 5, 6, 7, 8, 9, 10, 11 and 15, the PCR reactions were carried out in a 20 μL volume containing 2 μL of genomic DNA, 2 μL

of 1x PCR Buffer, 1.2 µL of 1.5mM $MgCl_2$, 2 µL of 0.25 mM dNTPs, 1 µL of 0.1 mM of each primers and 0.16 µL of 0.8 U of Ampli*Taq* Gold DNA polymerase (Applied Biosystems, USA). The tubes were placed on an Mastercycler Gradient (Eppendorf). The amplification condition was as follow: initial denaturation at 95°C for 15 minutes, followed by 35 cycles of the amplification process, which are denaturing 94°C for 30 seconds, annealing at 56°C for 1 minute and extension at 78°C for 1 minute, and the final extension step at 72°C for 7 minutes. After PCR procedure, 4 µL of PCR produces were checked by 1% (w/v) agarose gel electrophoresis. A standard 100 bp DNA ladder was used as a molecular weight marker.

For exon 12, 13 and 14, the PCR reactions were carried out in a 20 µL volume containing 2 µL of genomic DNA, 2 µL of 1x PCR Buffer, 1.2 µL of 1.5mM $MgCl_2$, 2 µL of 0.25 mM dNTPs, 1 µL of 0.1 mM of each primers and 0.16 µL of 0.8 U of Ampli*Taq* Gold DNA polymerase (Applied Biosystems, USA). The tubes were placed on an Mastercycler Gradient (Eppendorf). The amplification condition was as follow: initial denaturation at 95°C for 15 minutes, followed by 35 cycles of the amplification process, which are denaturing 94°C for 30 seconds, annealing at 62°C for 1 minute and extension at 78°C for 1 minute, and the final extension step at 72°C for 7 minutes. After PCR procedure, 4 µL of PCR produces were checked by 1% (w/v) agarose gel electrophoresis. A standard 100 bp DNA ladder was used as a molecular weight marker.

The optimized protocol, for the chemicals and temperature, respectively, used for the PCR are shown in **Table 2.4**, and **Table 2.5**.

Table 2.3 Intronal primer of *TP63*

Exon	Primer sequences for PCR (5' to 3')		Product size (bp)	References
	Forward	Reverse		
1	CCCTATTGCTTTTAGCCTCC	ACTGTGCTGACTAAACAAGG	281	Leoyklang et al., 2006
2	CTACATATATACCTGCATGG	AAAAACATGCCCTAGTAAGC	344	Leoyklang et al., 2006
3	AGCCTTGCTGACTTTGAAGC	CACATGACTGAAAAGACAGG	317	Leoyklang et al., 2006
3'	CTCCTCATGCCTATAGTTGG	GACCGAGAACCGCAAATACG	223	Leoyklang et al., 2006
4	ATGCATTCACCCATGGATGC	GAATCGCTAAACTGGGAAGG	437	Leoyklang et al., 2006
5	GTAAACAGGCAGCATGCAGC	AGTCTGAATCAGGTAGGTGG	401	Leoyklang et al., 2006
6	CACCAACATCCTGTTCATGC	GCTAGAAACATCCCTGTTGC	296	Leoyklang et al., 2006
7	AGAGGGAAGAACTGAGAAGG	CAGCCACGATTTCACTTTGC	256	Leoyklang et al., 2006
8	GGAAGTGGTAGATCTTCAGG	GCAGCTTCTCCAATATCACC	294	Leoyklang et al., 2006
9	GTGTTGCTGGTACTACTGTC	GACTAAGACACCTCCTTTCC	334	Leoyklang et al., 2006
10	ACTTCTAACAGTTCTACAGC	CTCATCAATCACCCTATTG	275	Leoyklang et al., 2006
11	CCATGTTTTAAACAGAGACC	CACAGAGTCTTGTCCTAAGC	313	Leoyklang et al., 2006
12	TTAACCAGACAAGATGGACC	CCCTTCCAACTGTTTTATGG	321	Leoyklang et al., 2006
13	CTTATCTCGCCAATGCAGTT	TACAAGGCGGTTGTCATCAG	238	Leoyklang et al., 2006
14	GGAATGATAGGATGCTGTGG	GCAGGAGTGCGCAGGAGTGC AAGATTAAGCAGGAGTGCTT	450	Leoyklang et al., 2006 Holder-Espinasse et al., 2007
15	CAGGCACTCTATTCTGTCTA	GGAAATACAACACACACACT	280	Leoyklang et al., 2006

Table 2.4 Optimized protocol for the chemicals used in the PCR procedure

Chemicals	**Concentration**	**Amount (µL)**
10x PCR Buffer	1x	2
25 mM Magnesium chloride ($MgCl_2$)	1.5 mM	1.2
2.5 mM deoxynucleotide triphosphate (dNTPs)	0.25 mM	2
10 µM Oligonucleotide primers (forward)	0.1 mM	1
10 µM Oligonucleotide primers (reverse)	0.1 mM	1
5 U/µL Ampli*Taq* Gold DNA polymerase	0.8 U	0.16
Genomic DNA	-	2
H_20	-	10.64
Total		**20**

Table 2.5 Optimized protocol for temperatures used in the PCR procedure

Exons	PCR Procedure				
	Initial Denaturation (°C for 15 min)	Amplification Process (35 Cycles)			Final Extension (°C for 7 min)
		Denaturing (°C for 30 s)	Annealing (°C for 1 min)	Extension (°C for 1 min)	
1	95.0	94.0	56.0	78.0	72.0
2			56.0		
3			56.0		
3'			56.0		
4			56.0		
5			56.0		
6			56.0		
7			56.0		
8			56.0		
9			56.0		
10			56.0		
11			56.0		
12			62.0		
13			62.0		
14			62.0		
15			56.0		

2.2.4.3 Agarose gel electrophoresis

The PCR products were checked by 1% (w/v) agarose gel electrophoresis. Agarose powder (0.35 mg) was boiled in 35 mL of 1x TBE buffer. Then 4 µL of the SYBR® Safe DNA gel stain 10,000x concentrate in DMSO (Invitrogen Inc., Carlsbad, Cal., USA) were added to the mixture. The gel was poured into the mold when the solution had cooled down to 60-70°C. After the gel was completely set (45-60 minutes at room temperature), the comb was gently withdrawn. Then sufficient 1x TBE buffer was poured into an electrophoresis tank to cover the gel to a depth of about 3-5 mm. Four microliters of PCR products were mixed with 1 µL of a 6x DNA loading dye and then were slowly loaded into slots in the gel by using an automatic micropipette. Electrophoresis was carried out at 115 V for 22 minutes using a Sub-Cell® GT Agarose Gel Electrophoresis system (BIO-RAD, Hercules, Cal., USA). After electrophoresis, DNA bands were visualized by UV light and documented. A standard 100-base-pair DNA ladder (GeneRuler™ 100 bp Plus DNA Ladder, ready-to-use, #SM0323, Fermentas UAB, Vilnius, Lithuania) was used as a molecular weight marker.

2.2.4.4 Purification of PCR products and DNA sequencing

PCR products were sent to Macrogen Inc. Seoul, Korea for purification and direct DNA sequencing.

2.2.5 Data analysis

To identify the pathogenic mutation of *TP63,* AB1 files of DNA sequences were analyzed by Sequencher 4.8 (Genecodes, Ann Arbor, Mich., USA). All mutations were confirmed by repeat sequencing. Forward and reverse sequences were analyzed and compared with an mRNA reference sequence. The position of a mutation corresponds to the coding sequence for the originally-published TAp63α isotype (GenBank accession number AF075430) **(Appendices A and B)** (Yang et al., 1998), which does not include the 39 additional codons for the amino-terminal end that were reported later by Hagiwara et al., 1999 (GenBank accession number AF091627). The changed nucleotides and amino acids were analyzed to the level of conservation of nucleotide and amino acids by using the University of California Santa Cruz (UCSC) website (http://genome.ucsc.edu).

To report or document the results, the positions of mutations were numbered, using the GenBank TAp63α mRNA sequence (GenBank accession number AF075430). That accession number is different from that used to report Single Nucleotide Polymorphisms (SNPs). The SNP reference sequence was linked to the *TP63* gene (gene ID: 8626) via the reference sequence: Contig NT_005612.15, mRNA NM_003722.4, protein NP_003713.3. There are a total of 1619 SNPs in the *TP63* gene. Eight of them are found within some of the coding regions of the *TP63* gene, and the remaining reported SNPs are found outside of the coding regions.

(www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?chooseRs=all&locusId=8626&mrna=NM_003722.4&ctg=NT_005612.15&prot=NP_003713.3).