CHAPTER II

RESEARCH DESIGN AND METHODS

2.1 RESEARCH DESIGN

To investigate mutations at codon 12 and codon 13 of K-ras gene, we amplified DNA samples by polymerase chain reaction and detected mutations using restriction fragment length polymorphism (RFLP) technique. Since there is no natural restriction site within these codons, we created restriction site on PCR products by using special primers which created restriction sites only for normal allele. To detect mutations in codon 12, the special primer created restriction site for *BstN*I (CCA/TGG); and for codon 13, *Hae*III (GGCC). Furthermore, we confirmed our results by DNA sequencing. The diagrams below show the procedure to detect mutations at codon 12 and codon 13, respectively.

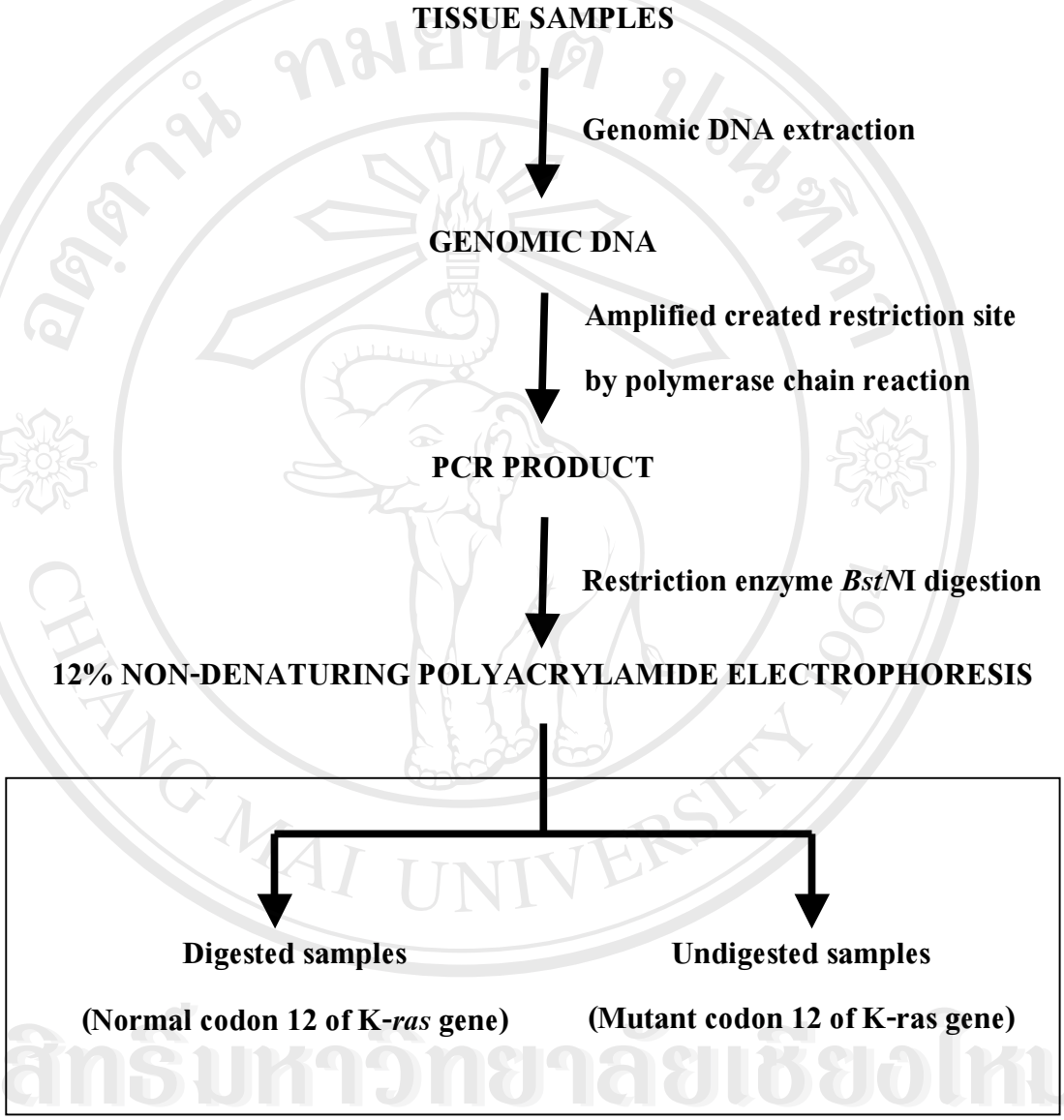

Figure 14 The procedure of determination of the mutation at codon 12 of the K-*ras* gene from the Thai ovarian cancer tissue samples by using amplified created restriction site method with polymerase chain reaction - restriction fragment length polymorphism.

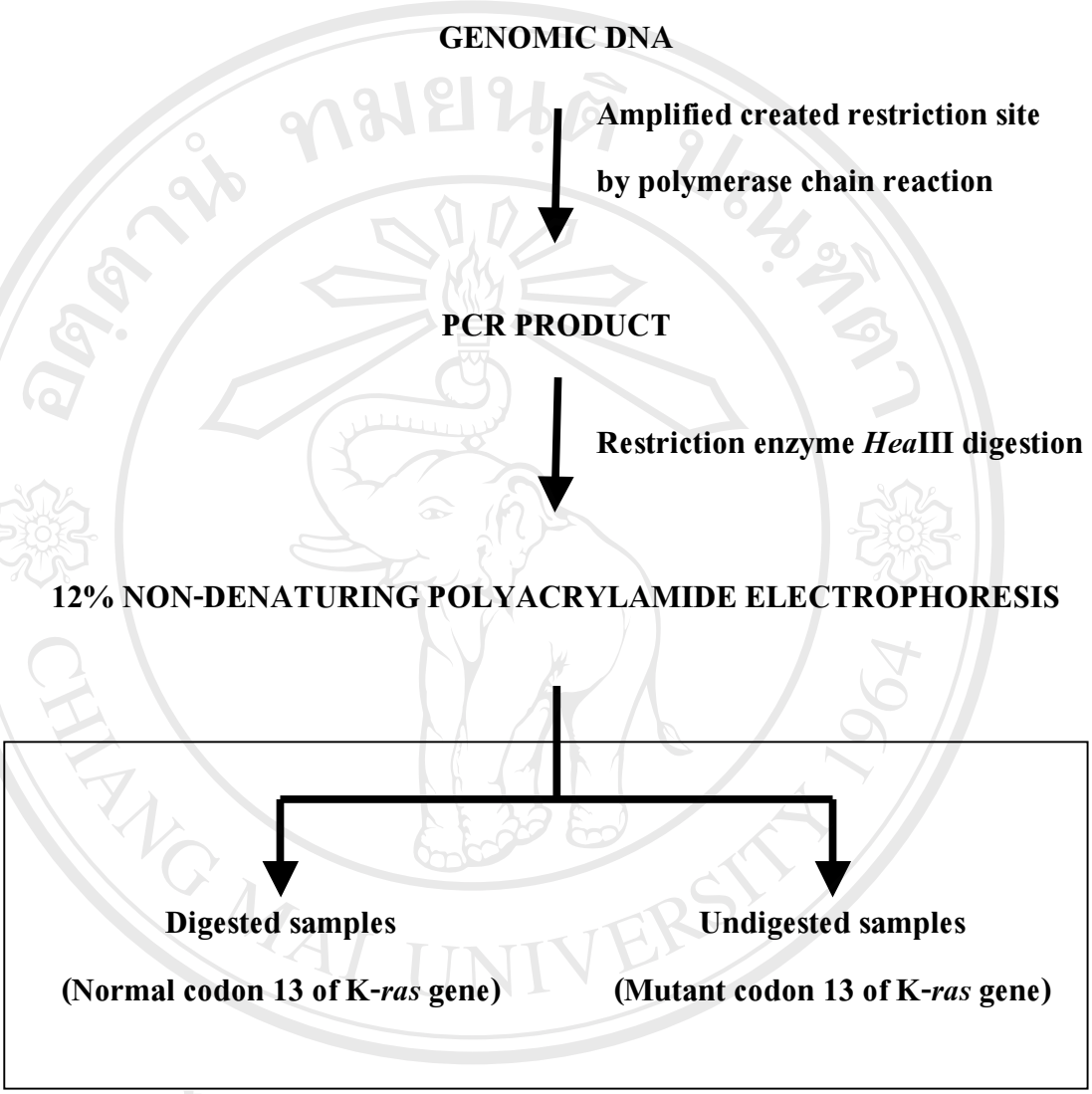

Figure 15 The procedure of determination of the mutation at codon 13 of the K-*ras* gene from the Thai ovarian cancer tissue samples by using amplified created restriction site method with polymerase chain reaction - restriction fragment length polymorphism.

2.2 METHODS

2.2.1 Tissue Samples

The ovarian tumor specimens were obtained from 82 patients who were diagnosed as ovarian cancer from the Department of Pathology, Faculty of Medicine, Maharaj Nakorn Chiang Mai hospital, Chiang Mai University, Thailand, during the period between January 2004 to March 2005. Histological features of these tissues and the type of cancer were indicated by pathologist Associate Professor Sumalee Siriaungkul as follows: benign 4 cases, tumor of low malignancy 23 cases, and adenocarcinomas 39 cases, that 60 epithelial tumors, 5 sex cord stromal cell tumors, 5 germ cell tumors, and 12 metastatic tumors. The 60 epithelial tumors included 2 mucinous cytsadenomas, 16 mucinous tumor of low malignant potential (LMP) **(Figure 16a)**, 4 mucinous adenocarcinomas **(Figure 16b)**, 5 serous tumor of LMP **(Figure 16c)**, 13 serous adenocarcinomas **(Figure 16d)**, 1 endometrioid tumor of LMP **(Figure 16e)**, 10 endometrioid adenocarcinomas **(Figure 16f)**, 6 clear cell adenocarcinomas **(Figure 16g)**, and 3 mix epithelial tumors. The 5 sex cord stromal cell tumors included 4 granulosa cell tumors and 1 unclassified sex-cord tumor. The 5 germ cell tumors included 3 immature teratomas, 1 squamous cell carcinoma arising in teratoma and solid mature teratoma. The 12 metastatic tumors included 7 matastatic adenocarcinoma, 3 matastatic signetring cell carcinoma, 1 metastatic SCC from Cevix and matastatic ductal carcinoma. The average age of this group of patients was 47.61 $\pm$ 12.10 years (ranging from 15 to 76 years). Clinicopathologic data are showed in **Table 1**. All tumor specimens were submerged in 10 ml of sterile normal saline and kept at -20°C before DNA extraction.

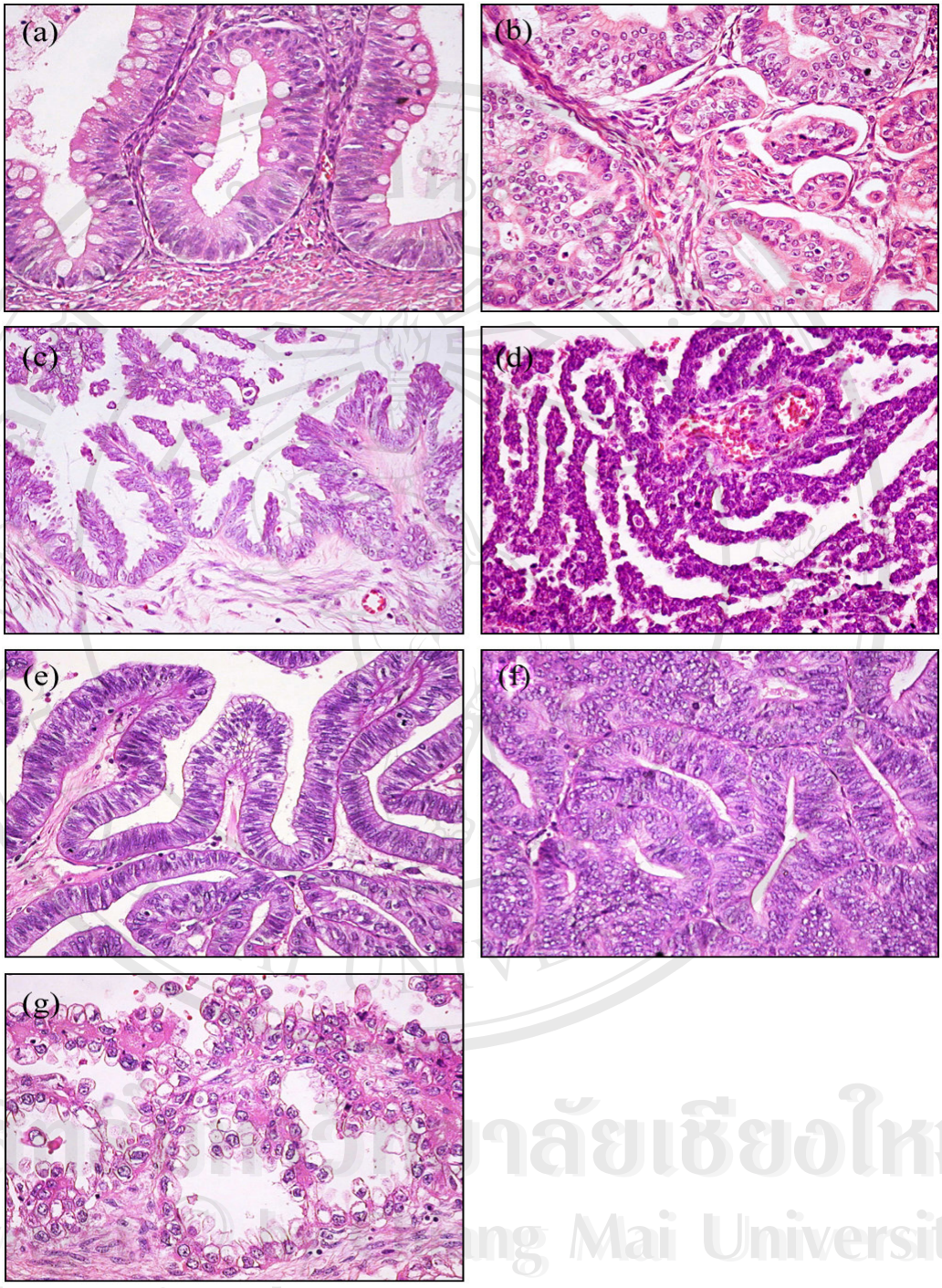

Figure 16 Histology of ovarian cancer. Hematoxylin and eosin stained stained section (x200) with **(a)** mucinous tumors of LMP, **(b)** mucinous adenocarcimas **(c)** serous tumors of LMP, **(d)** serous adenocarcimas, **(e)** endometrioid tumors of LMP, **(f)** endometrioid adenocarcimas, and **(g)** clear cell adenocarcimas.

Table 1 Clinicopathologic features data of Thai ovarian cancer patients.

Case number	DNA code	Age (year)	Histologic subtype	Tumor classification		
				Benign	LMP	Malignant
1	OT 001	47	Serous adenocarcinoma			3
2	OT 002	49	Endometrioid adenocarcinoma			1
3	OT 003	49	Mucinous tumor of LMP		Low	
4	OT 004	48	Serous adenocarcinoma			3
5	OT 005	62	Clear cell adenocarcinoma			3
6	OT 006	34	Immature teratoma			1
7	OT 007	53	Clear cell adenocarcinoma			3
8	OT 008	59	Serous adenocarcinoma			2
9	OT 009	54	Mucinous tumor of LMP		High	
10	OT 010	48	Mucinous adenocarcinoma			2
11	OT 011	50	Mucinous tumor of LMP		Low	
12	OT 012	76	Serous tumor of LMP		Low	
13	OT 013	43	Granulosa cell tumor			
14	OT 014	29	Metastatic adenocarcinoma			
15	OT 015	17	Metastatic adenocarcinoma			
16	OT 016	53	Endometrioid adenocarcinoma			3
17	OT 017	15	Mucinous adenocarcinoma			1
18	OT 018	56	Endometrioid adenocarcinoma			1
19	OT 019	53	Metastatic adenocarcinoma			
20	OT 020	40	Endometrioid adenocarcinoma			3
21	OT 021	50	Clear cell adenocarcinoma			3
22	OT 022	51	Endometrioid adenocarcinoma			3
23	OT 023	37	Serous tumor of LMP with focal CA			1

Table 1 (continued) Clinicopathologic features data of Thai ovarian cancer patients.

Case number	DNA code	Age (year)	Histologic subtype	Tumor classification		
				Benign	LMP	Malignant
24	OT 024	63	Mucinous cystadenoma	Benign		
25	OT 025	50	Mucinous cystadenoma	Benign		
26	OT 026	51	Mixed epithelial tumor of LMP		High	
27	OT 027	47	Mixed epithelial carcinoma			2
28	OT 028	42	Mucinous tumor of LMP		High	
29	OT 029	31	Metastatic adenocarcinoma			
30	OT 030	45	Metastatic SCC from Cevix			
31	OT 031	61	Granulosa cell tumor			
32	OT 032	50	Endometrioid adenocarcinoma			3
33	OT 033	64	Serous adenocarcinoma			2
34	OT 034	38	Granulosa cell tumor			
35	OT 035	46	Serous tumor of LMP*			
36	OT 036	73	Mucinous tumor of LMP		Low	
37	OT 037	46	Clear cell adenocarcinoma			
38	OT 038	17	Mucinous tumor of LMP		High	
39	OT 039	42	Metastatic adenocarcinoma			
40	OT 040	45	Endometrioid adenocarcinoma			2
41	OT 041	41	Mucinous adenocarcinoma			1
42	OT 042	47	Mucinous tumor of LMP *		High	
43	OT 043	68	Squamous cell carcinoma/teratoma			1
44	OT 044	61	Endometrioid adenocarcinoma			3
45	OT 045	58	Mixed epithelial carcinoma			2
46	OT 046	46	Mucinous tumor of LMP		Low	

* tumor with microinvasion

Table 1 (continued) Clinicopathologic features data of Thai ovarian cancer patients.

Case number	DNA code	Age (year)	Histologic subtype	Tumor classification		
				Benign	LMP	Malignant
47	OT 047	41	Mucinous tumor of LMP		Low	
48	OT 048	38	Solid mature teratoma	Benign		
49	OT 049	38	Endometrioid tumor of LMP *		Low	
50	OT 050	56	Adenocarcinoma (1°vs 2°)			3
51	OT 051	63	Endometrioid adenocarcinoma			2
52	OT 052	62	Mucinous tumor of LMP		Low	
53	OT 053	31	Matastatic signetring cell carcinoma			
54	OT 054	42	Mucinous tumor of LMP *		High	
55	OT 055	42	Endometrioid adenocarcinoma			1
56	OT 056	48	Serous tumor of LMP		Low	
57	OT 057	45	Mucinous tumor of LMP*		High	
58	OT 058	44	Matastatic signetring cell carcinoma			
59	OT 059	49	Serous adenocarcinoma			2
60	OT 060	44	Matastatic adenocarcinoma			
61	OT 061	55	Serous adenocarcinoma			3
62	OT 062	54	Serous adenocarcinoma			3
63	OT 063	23	Serous tumor of LMP*			
64	OT 064	49	Clear cell adenocarcinoma			3
65	OT 065	50	Mucinous tumor of LMP		High	
66	OT 066	49	Serous adenocarcinoma			3
67	OT 067	57	Clear cell adenocarcinoma			3
68	OT 068	55	Serous adenocarcinoma			3
69	OT 069	30	Mucinous tumor of LMP		High	

* tumor with microinvasion

Table 1 (continued) Clinicopathologic features data of Thai ovarian cancer patients.

Case number	DNA code	Age (year)	Histologic subtype	Tumor classification		
				Benign	LMP	Malignant
70	OT 070	22	Immature teratoma			3
71	OT 071	65	Granulosa cell tumor			
72	OT 072	47	Mucinous tumor of LMP		Low	
73	OT 073	48	Mucinous adenocarcinoma			2
74	OT 074	44	Matastatic ductal carcinoma			
75	OT 075	50	Matastatic adenocarcinoma			
76	OT 076	53	Serous adenocarcinoma			2
77	OT 077	59	Unclassified sex-cord tumor			
78	OT 078	31	Immature teratoma			
79	OT 079	66	Mucinous tumor of LMP		High	
80	OT 080	62	Serous adenocarcinoma			2
81	OT 081	42	Serous adenocarcinoma			2
82	OT 082	45	Matastatic signetring cell carcinoma			

2.2.2 Genomic DNA Preparation

Genomic DNA preparation was modified from the method described by Miller et al. (1988). First, the tissue was cut and ground to fine powder. Then the intact red blood cells were lysed with 10 ml of Red Cell Lysis buffer. The mixture was subsequently centrifuged at 3,000 rpm for 10 minutes and the supernatant was discarded. The pellets were ground again and resuspended in 8 ml Nuclei Lysis buffer. After mixing gently with 0.5 ml of 10% sodium dodecyl sulfate (SDS), the cells were lysed and the mixtures became viscous. The mixture was added with 1.0 ml proteinase K solution (1 mg/ml) and incubated in shaking water bath at 56°C overnight. The digested protein was separated by salting out with 5.0 M NaCl at the ratio of 1 to 4 volumes of the mixture. To complete protein precipitation, the mixture was kept at 4°C for 10 minutes before being centrifuged at 4°C, 5000 rpm for 15 minutes. The clear supernatant was subsequently transferred to new tube and DNA was precipitated with 2.5 volumes of ice-cold absolute ethanol. The white fluffy DNA was then hooked out, rinsed 3 times with 0.5 ml of 70% ethanol to wash the excess salt, and kept dry at room temperature for 2 hours. Finally, the DNA was dissolved in 0.5 ml of 10 mM Tris-base and 0.1 mM EDTA (TE) buffer and was ready to use for the experiment.

The concentration of DNA was determined by spectrophotometry. An OD of 1 at wavelength of 260 nm corresponds to approximately 50 μg/ml for double-stranded DNA. The obtained DNA was diluted to 50 times in TE buffer before measuring. The concentration of DNA was calculated by using the formula as below:

Concentration of DNA (μg/ml) = OD 260 x 50 x dilution factor

The quality of the DNA is essential to the overall success of the analysis. OD 260 is frequently used to measure DNA concentration and OD 280 is used to measure protein concentration. For further analysis it is imperative that the DNA extracted should have high purity, displaying ratio of OD 260/OD 280 values between 1.7 to 1.9. Smaller ratios usually indicates contamination by protein or organic chemicals.

2.2.3 Created Restriction Site DNA Amplification

2.2.3.1 Primer Selection

For K-*ras* gene, the cytogenetic map position of the gene was found to be 12p12.1-p11.1. To determine the mutation at codon 12 and codon 13 on exon I of the K-*ras* gene, the created restriction site DNA amplification was employed **(Figure 17)**. For codon 12, the forward primer was designed to create an artificial *Bst*NI (CCA/TGG) restriction site in the normal allele by substituting G with C in the second position of codon 11 **(Figure 18)**. For codon 13**,** the mismatched reverse primer was created a restriction site for *Hae*III (GGCC) in the normal allele by substituting G with C in the first position of codon 14 **(Figure 19)**. Below are position and sequence of primers used in this study:

K-*ras* codon 12

Forward primer (nt 99-128)

5'-ACTGAATATAAACTTGTGGTAGTTGGA**C**CT-3'

Reverse primer (nt 232-260)

5'-CTGTATCAAAGAATGGTCCTGCACCAGTA-3'

K-*ras* codon 13

Forward primer (nt 1-30)

5'-GTACTGGTGGAGTATTTGATAGTGTATTAA-3'

Reverse primer (nt 134-159)

5'-GTATCGTCAAGGCACTCTTGCCTA**G**G-3'

The underlined bases represent mismatches from the K-*ras* DNA sequence. The normal sequence of exon I of K-*ras* gene obtained from Gene Bank ID: L00045 shown in **Figure 18** and **19.** The primers used for the amplification of codon 12 and codon 13 were reported by A. Hatzaki et al**.** (Hatzaki, Razi et al. 2001).

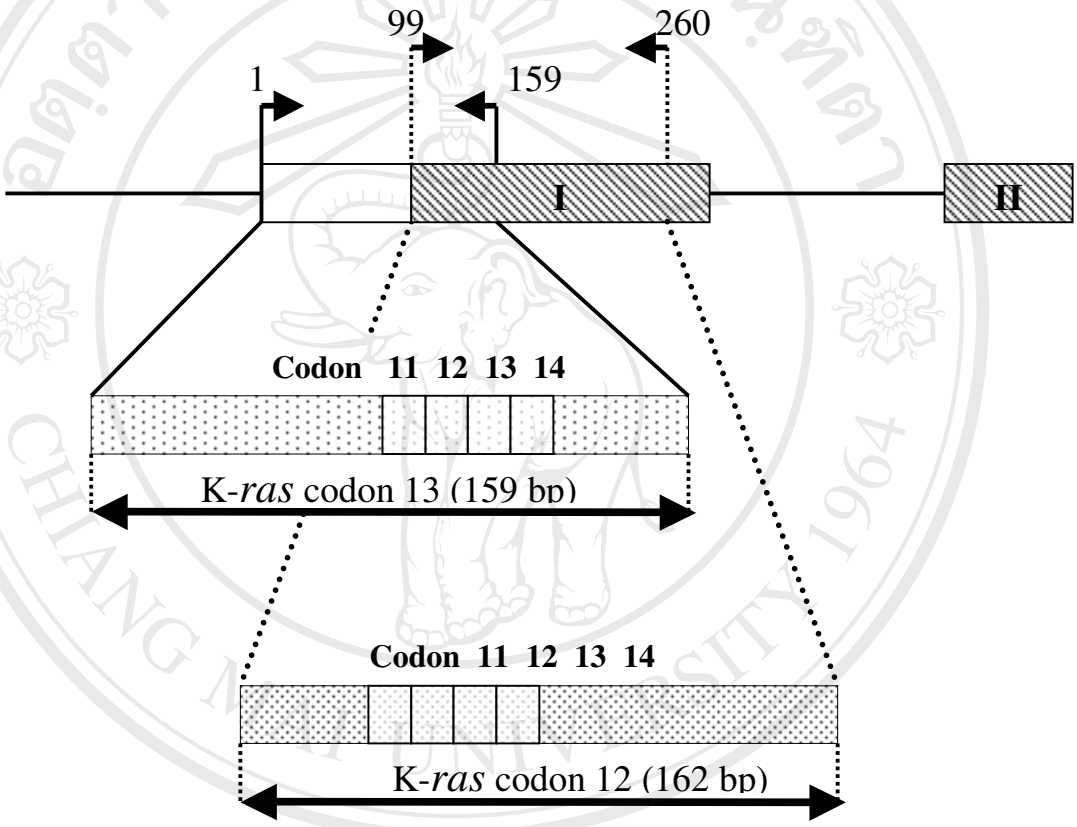

Figure 17 The location of primers for determination the mutation at codon 12 and codon 13 on exon I of the K-*ras* gene using the amplified created restriction site method with polymerase chain reaction technique. The symbol → indicates forward primer and ← indicates reverse primer. The □ box represents non-coding sequence and ▨ box represents coding sequence

1 GTACTGGTGG AGTATTTGAT AGTGTATTAA CCTTATGTGT GACATGTTCT

51 AATATAGTCA CATTTTCATT ATTTTTATTA TAAGGCCTGC TGAAAATGAC

Forward primer ***AC***

Codon 11 12 13 14

101 TGAATATAAA CTTGTGGTAG TTGGAGCT**GG T**GGCGTAGGC AAGAGTGCCT

TGAATATAAA CTTGTGGTAG TTGGAC<u>C</u>CT

151 TGACGATACA GCTAATTCAG AATCATTTTG TGGACGAATA TGATCCAACA

201 ATAGAGGTAA ATCTTGTTTT AATATGCATA TTACTGGTGC AGGACCATTC

Reverse primer ***TACTGGTGC AGGACCATTC***

251 TTTGATACAG ATAAAGGTTT CTCTGACCAT TTTCATGAGT

TTTGATACAG

Figure 18 The nucleotide sequence of exon I in K-*ras* gene obtain from Gene Bank ID: L00045 of normal human K-*ras* gene. The underlined bases represent forward primer mismatches for amplifying created restriction site codon 12.

1 GTACTGGTGG AGTATTTGAT AGTGTATTAA CCTTATGTGT

Forward primer ***GTACTGGTGG AGTATTTGAT AGTGTATTAA***

41 GACATGTTCT AATATAGTCA CATTTTCATT ATTTTTATTA TAAGGCCTGC

Codon 11 12 13 14

91 TGAAAATGAC TGAATATAAA CTTGTGGTAG TTGGAGCTGG T**GG**CGTAGGC

Reverse primer ***CCTAGGC***

141 AAGAGTGCCT TGACGATACA GCTAATTCAG AATCATTTTG TGGACGAATA

AAGAGTGCCT TGACGATAC

191 TGATCCAACA ATAGAGGTAA ATCTTGTTTT AATATGCATA TTACTGGTGC

241 AGGACCATTC TTTGATACAG ATAAAGGTTT CTCTGACCAT TTTCATGAGT

Figure 19 The nucleotide sequence of exon I in K-*ras* gene obtain from Gene Bank ID: L00045 of normal human K-*ras* gene. The underlined bases represent reverse primer mismatches for amplifying created restriction site codon 13.

2.2.3.2 PCR Optimization

Efficiency and specificity of amplification are highly dependent on the nature of the template DNA, primers, annealing temperature, magnesium chloride, and dNTPs concentration. Therefore, the optimization of reaction conditions is frequently necessary for each specific PCR products. To optimize our PCR conditions, annealing temperature was varied within the range of several temperatures below and above the calculated Tm, deoxynucleotide triphosphates (dNTPs) concentration was varied from 50 μM to 250 μM, primer concentration was varied from 100 μM to 500 μM, and magnesium chloride ($MgCl_2$) concentration was varied from 1.0 to 3.0 mM. The optimized conditions resulted in the efficient amplification without non-specific amplified products.

The procedures and optimized protocols are as follows:

For codon 12, the 50 μl reaction mixture contained 1 μg of extracted DNA, 1X buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl and 0.1% Triton® X-100), 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 2 units of *Taq* polymerase (Promega), and 0.3 μM of primers. The tubes were then placed on a Mastercycle gradient thermal cycler (Eppendorf). The temperature profile was as follows: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of the amplification process, which are denaturation at 94°C for 40 seconds, annealing at 60°C for 30 seconds and extension at 74°C for 30 seconds, and the final extension step at 74°C for 7 minutes.

For codon 13, the 50 μl reaction mixture contained 1 μg of extracted DNA, 1X buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl and 0.1% Triton® X-100), 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 2 units of *Taq* polymerase (Promega), and 0.2 μM of primers. The tubes were then placed on a Mastercycle gradient thermal cycler (Eppendorf). The temperature profile was as follows: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of the amplification process, which are denaturation at 94°C for 40 seconds, annealing at 63°C for 30 seconds and extension at 74°C for 30 seconds, and the final extension step at 74°C for 7 minutes.

After amplification, a 2 μl aliquot was analyzed by electrophoresis on a 2% (w/v) agarose gel and then was stained with ethidium bromide. The standard 100-base pairs DNA ladder was used as molecular weight marker.

2.2.3.3 Agarose Gel Electrophoresis

The PCR products were checked by 2% (w/v) agarose gel electrophoresis. The 0.3 mg of agarose powder was boiled in 15 ml of 0.5X TBE buffer and the gel were poured into the mold when the solution cooled down to 60-70°C. After the gel was completely set (45 - 60 minutes at room temperature), gently withdrew the comb. Then 0.5X TBE buffer was poured into electrophoresis tank in the amount of buffer that could cover the gel to a depth of about 3 to 5 mm. Two microliters of PCR products were mixed with 2 µl of a loading buffer and then were slowly loaded into the slots by using automatic micropipette. Electrophoresis was carried out at 100 V for 30 minutes. After electrophoresis, the gel was stained by submerging in 0.5 µg/ml ethidium bromide solution for 15-20 minutes and then was destained twice in distilled water for 5-10 minutes. The DNA bands were visualized by UV light and documented by using a Bio-Rad gel doc 1000 system. The standard 100-base pairs DNA ladder was used as molecular weight maker.

2.2.3.4 Purification of PCR Products

The PCR products were purified before any step of restriction enzyme digestion in order to remove excess PCR primers, dNTPs, enzyme, and buffer component from PCR amplification. First, the 5.0 M NaCl at the ratio of 1 to 10 volumes of the mixture was added. Then cold absolute ethanol at the ratio of 2.5 to 1 volumes of the mixture was dispensed in each tube, mixed thoroughly and kept at –20°C overnight to ensure the precipitation of PCR products. The precipitated PCR products were recovered by centrifugation at 12,000 rpm for 30 minutes. The supernatant was removed and the pellet was rinsed 2-3 times with 150 µl of 70% ethanol to wash the excess salt. The pellet was left to dry at room temperature and redissolved with 20 µl sterile deionized water.

2.2.3.5 Restriction Enzyme Digestion

The expected sizes of the PCR products are 162 base pairs and 159 base pairs for codon 12 and codon 13, respectively. For codon 12, the 10 µl restriction reaction mixture contained 3 µl of purified PCR product, 2.5 units of *Bst*NI, 1X Buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol (pH 7.9)), 1µl of 100 µg/ml BSA and sufficient amount of

sterile deionized water to make a final volume to 10 µl. The reaction mixture was then incubated at 60°C overnight before separation by 12% (w/v) non-denaturing polyacrylamide gel. For codon 13, the 10 µl restriction reaction mixture contained 3 µl of purified PCR product, 2.5 units of *Hae*III, 1X Buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol (pH 7.9)), and sufficient amount of sterile deionized water to make a final volume to 10 µl. The reaction mixture was then incubated at 37°C overnight before separation by 12% (w/v) non-denaturing polyacrylamide gel.

2.2.3.6 Polyacrylamide Gel Electrophoresis

The products of restriction enzyme reactions were analyzed by 12% non-denaturing polyacrylamide gel electrophoresis. The gel electrophoresis was performed using a Bio-Rad Mini-Protean® II 2-D cell. The 5-ml acrylamide solution contained 2.0 ml 30% polyacrylamide solution, 1.95 ml of deionized water, 1.0 ml of 5X TBE, and 50 µl of 10% ammonium persulfate. A 5.0-µl TEMED (N, N, N', N'-tetramethylethylenediamine) was added to the solution and mixed thoroughly right before pouring the gel solution into the gel casting unit. The comb (5 mm-width and 0.75 mm-thick) was inserted and the gel was let to polymerize for 45 minutes.

After the gel was completely polymerized, the gel plate was assembled to the electrophoresis unit. Then 1X TBE buffer was poured into electrophoresis tank in the amount of buffer that could cover the tank and the gel plate edge was submerged. Whole restriction enzyme digestion product or 3 µl of purified PCR product was mixed with 3 µl loading buffer and was slowly loaded into the slots using an automatic micropipettor. Electrophoresis was carried on 100 V for 2 hours. After electrophoresis, the gel was stained in 0.5 µg/ml ethidium bromide solution for 20 minutes and then destained in distilled water for 10 minutes thrice. The DNA bands were visualized under UV light and documented by using a Bio-Rad gel doc 1000 system. The standard 100-base pairs DNA ladder was used as molecular weight maker.

For codon 12, the size of non-digested PCR product is 162 bp. With *Bst*NI digestion, which recognizes the sequence CC$_{A/T}$GG amplified from normal allele, two fragments of 133 bp and 29 bp were expected. Therefore, normal allele would result in two fragments of 133 bp and 29 bp, while mutated allele would remain the whole intact 162 bp PCR product after *Bst*NI digestion.

For codon 13, the size of non-digested PCR products is 159 bp. With *Hae*III digestion, which recognizes the sequence GGCC amplified from normal allele, three fragments of 85, 48 and 26 bp were expected. Therefore, normal allele would result in three fragments of 85, 48, and 26 bp, while mutated allele (at codon 13) would produce the two fragments of 85 and 74 bp after *Hae*III digestion. **(Figure 20)**.

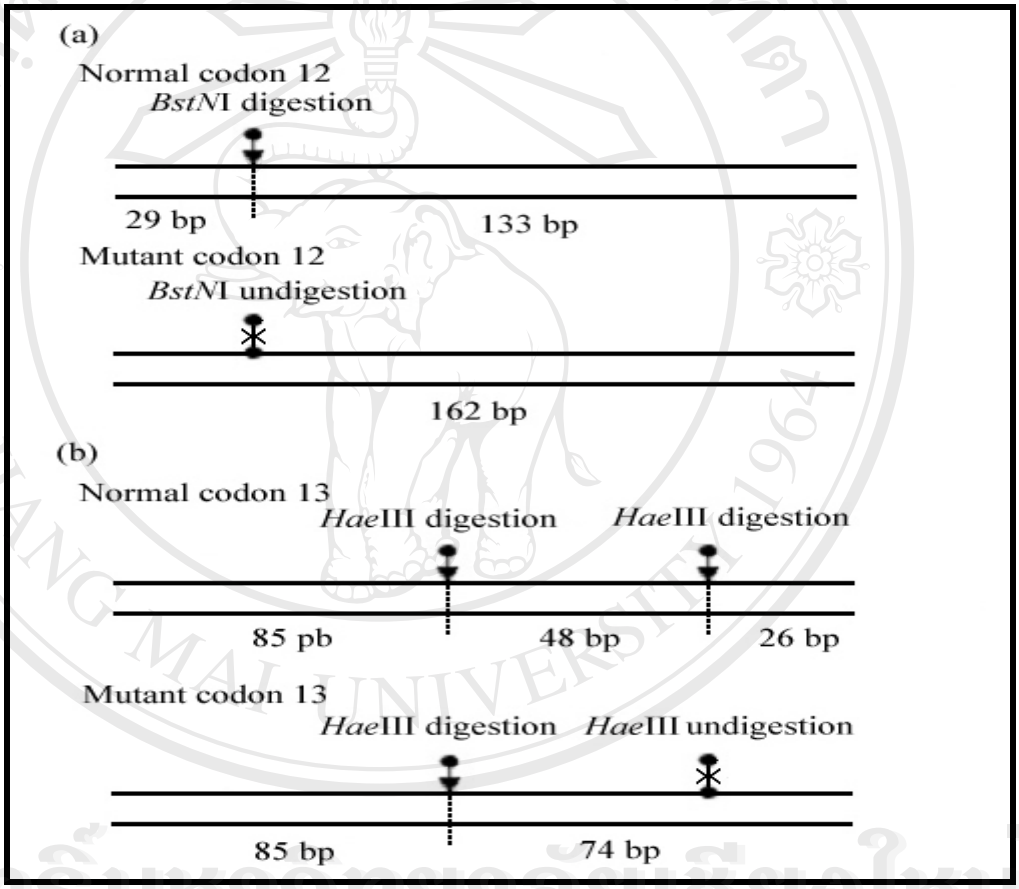

Figure 20 Site and size of expected DNA fragments after restriction enzyme digestion. (a) For codon 12 mutation detection. (b) For codon 13 mutation detection. The symbol ✳ indicates undigested restriction site and ▼ indicates digested restriction site.

2.2.4 DNA Sequencing

All mutations identified by the amplified created restriction site method with PCR-RFLP analysis were confirmed by DNA sequencing. The new pair of primers were used for amplifying DNA fragment which covered codon 12 and codon 13 of K-*ras* gene. The amplified PCR products were purified with low melting point agarose gel electrophoresis and a QAIquick gel extraction purification kit (QAIGEN). After purification, direct sequencing was carried out by the dideoxynucleotides chain termination method using a BigDye® Terminator Cycle Sequencing Kit, v3.1 (Applied Biosystems), according to the manufacturer's protocols. Sequencing reaction of PCR amplified fragments were performed on a Mastercycle gradient thermal cycler (Eppendorf). The sequence reaction products were analyzed by using a Perkin Elmer ABI Prism 310 Genetic Analyzer (Applied Biosystems, Tokyo, Japan) **(Figure 21)**.

2.2.4.1 Primer Designs

The primers were designed to cover codon 12 and codon 13 of K-*ras* gene. The primers below were expected to give the PCR product of 260 bp. The locations of primers and codon 12 and codon 13 are shown in Figure 2-9.

Forward primer (nt 1-30)

5'-GTACTGGTGGAGTATTTGATAGTGTATTAA-3'

Reverse primer (nt 232-260)

5'-CTGTATCAAAGAATGGTCCTGCACCAGTA-3'

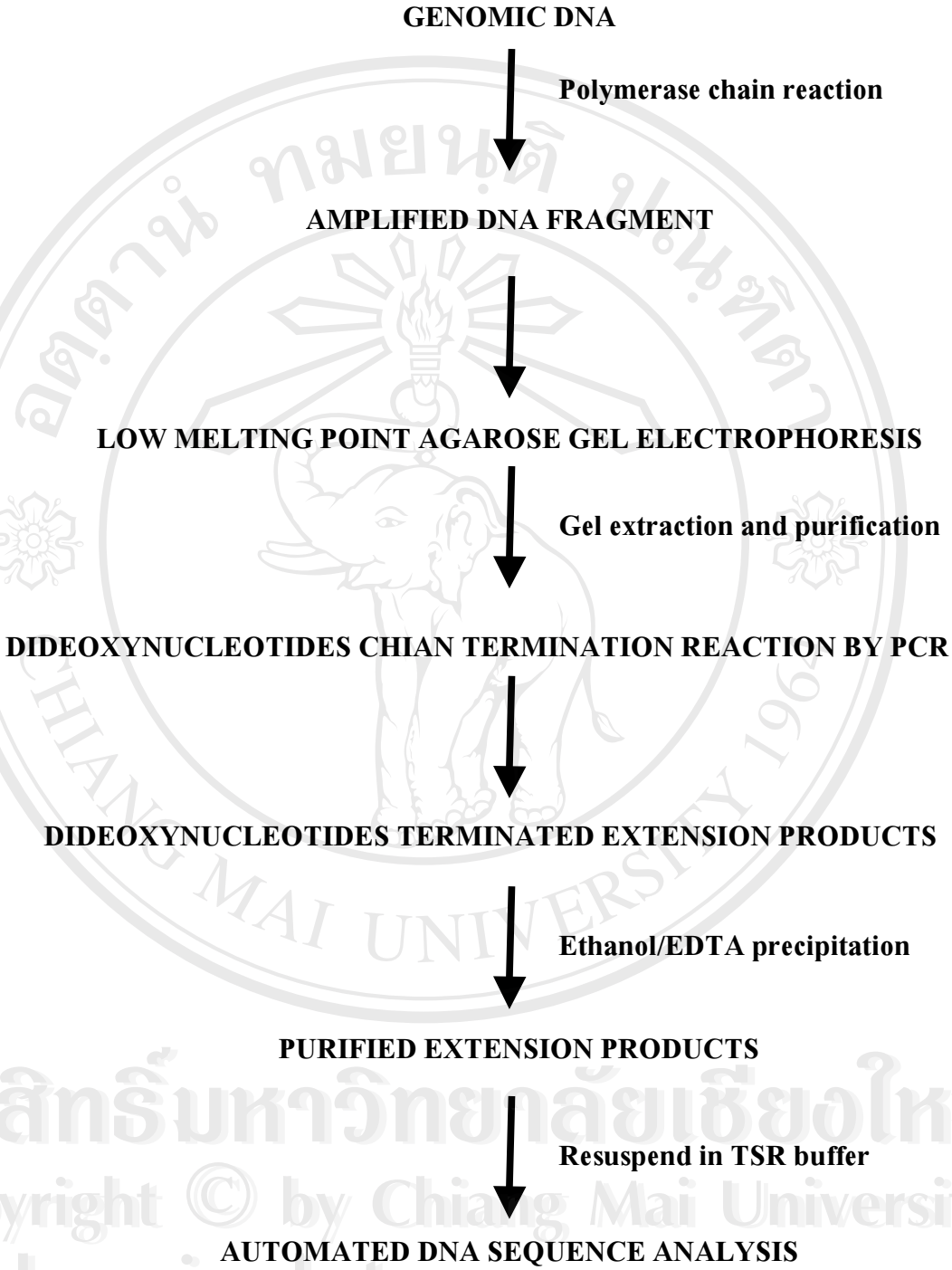

Figure 21 The procedure for determinating the mutation at codon 12 and codon 13 on exon I of K-*ras* gene from the Thai ovarian cancer tissue samples by DNA sequencing using dideoxynucleotides chain termination.

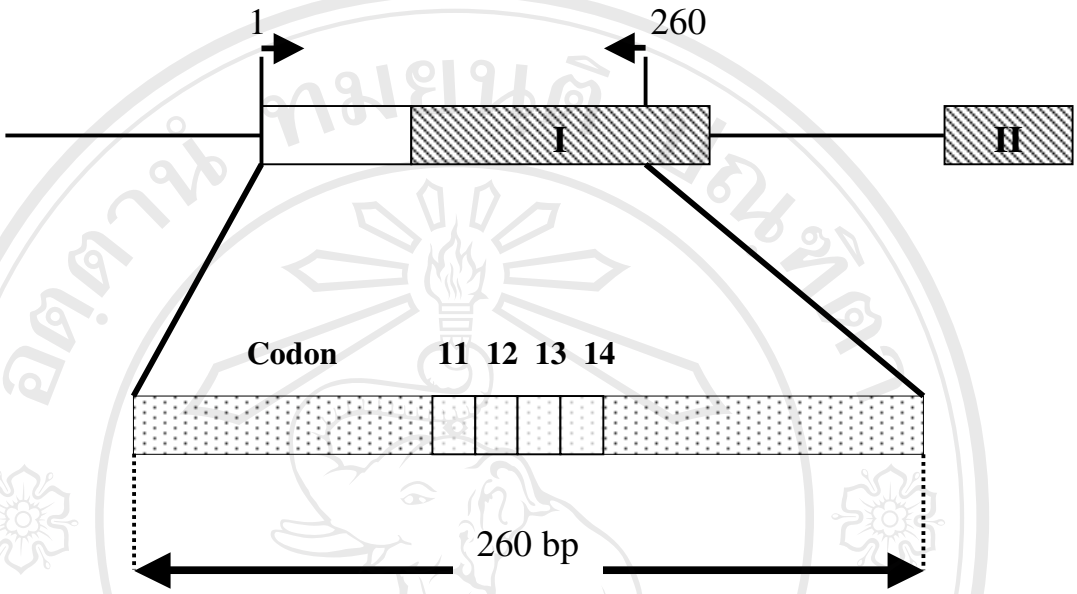

Figure 22 The location of primers for amplified DNA fragments covering codon 12 and codon 13 on exon I of K-*ras* gene by polymerase chain reaction. The symbol ➔ indicates forward primer and ➔ indicates reverse primer. The □ box represents non-coding sequence and ▨ box represents coding sequence

2.2.4.2 PCR Optimization

To optimize this PCR condition, annealing temperature was varied within the range from 56.0°C to 69.0°C, deoxynucleotide triphosphates (dNTPs) concentration was varied from 50 μM to 250 μM, primer concentration was varied from 100 μM to 500 μM, and magnesium chloride ($MgCl_2$) concentration was varied from 1.0 to 3.0 mM. The optimized conditions resulted in the efficient amplification of the 260-bp DNA without non-specific amplified product.

The procedures and optimized protocol was:

The 50 μl reaction mixture contained 1 μg of extracted DNA, 1X buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl and 0.1% Triton® X-100), 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 2 units of

Taq polymerase (Promega), and 0.2 μM of each primer. The tubes were then placed on a Mastercycle gradient thermal cycler (Eppendorf). The temperature profile was as follows: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of the amplification process, which are denaturation at 94°C for 1 minutes, annealing at 60°C for 1 minutes and extension at 74°C for 1 minutes, and the final extension step at 74°C for 7 minutes.

2.2.4.3 Purification of PCR Product

The PCR product was purified by 1% (w/v) low melting point agarose gel electrophoresis. The 0.18 mg of the low melting point agarose powder was boiled in 18 ml of 0.5X TBE buffer. When the solution cooled down to 60-70ºC, ethidium bromide was added to a final concentration of 0.5 μg/ml. The gel was then poured into the mold. After the gel was completely set (45 - 60 minutes at cool temperature), gently withdrew the comb. The 0.5X TBE buffer was poured into electrophoresis tank in the amount of buffer that could cover the gel to a depth of about 3 to 5 mm. The whole 50-μl PCR product was mixed with 5 μl of loading buffer and then was slowly loaded into the gel slot by using automatic micropipette. Electrophoresis was carried out with 100 V for 30 minutes at cool temperature to ensure that the gel does not melt during the run. The standard 100-base pairs DNA ladder was used as molecular weight marker. After electrophoresis, the PCR product was visualized by UV light and the band was cut out with a clean, sterile scalpel. The gel fragment was purified using QAIquick gel extraction purification kit (QAIGEN).

2.2.4.4 Dideoxynucleotides Chain Termination Reaction

After PCR products were purified by using QAIquick gel extraction purification kit (QAIGEN), the concentration of PCR products were measured by spectrophotometry at 260 nm. The 20-μl dideoxynucleotides chain termination reaction contained 50 ng of purified PCR products, 3.2 pmole of forward primer, 8 μl BigDye® Sequencing Ready Reaction Buffer and sterile deionized-distilled water. This sequencing reaction of mixture was performed on a Mastercycle gradient thermal cycler (Eppendorf). The condition for dideoxynucleotides chain termination reaction were programmed to 96°C for 1 minutes, followed by repeating 25 cycles of

96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. The dideoxynucleotides extension products were stored at 8°C until purification.

2.2.4.5 Purification of the Dideoxynucleotide Terminated Extension Products

The extension products were precipitated with ethanol/EDTA precipitation. The entire content of extension product was added with 5 µl of 125 mM EDTA and 60 µl of cold absolute ethanol, then mixed gently and incubated on ice for at least 15 minutes to precipitate the extension products. The tube was spun in microcentrifuge at 13,000 rpm for 45 minutes and the supernatant were carefully removed without disturbing the pellet. The pellets were rinsed twice with 125 µl of 70% ethanol and centrifuged at 4°C, 13,000 rpm for 20 minutes, and the supernatant was carefully removed without disturbing the pellet. The pellet was kept dry at room temperature until it was completely dry. The purified extension products were stored at -20°C until use.

2.2.4.6 Automated DNA Sequence Analysis

The purified extension products were resuspended in 25 µl of template suppression reagent (TSR), then were mixed thoroughly and spun down. The samples were heated at 95°C for 5 minutes, followed by chilling on ice for 10 minutes. The samples were then mixed thoroughly before transferring to a Perkin Elmer ABI Prism 310 Genetic Analyzer. The raw data from capillary electrophoresis on the Perkin Elmer ABI Prism 310 Genetic Analyzer were collected and analyzed by Macintosh-based software (Sequencing Analysis Software Version 2.1.1). The bases at codon 12 and codon 13 of K-*ras* gene were manually compared with the normal sequences (The normal nucleotide sequence in exon I of K-*ras* gene were derived form Gene Bank ID: L00045).

2.2.5 Data Analysis

Statistical differences between mutation and normal groups were presented as percentage. The determination of K-*ras* gene mutation at codon 12 and codon 13 were characterized upon the length of PCR fragment after digested with restriction enzyme on gel electrophoresis. Normal sequence at codon 12 produced a digested product of 133 bp and 29 bp in length, whereas the

PCR product from mutant sequence was a 162 bp undigested product. For detection of normal at codon 13, the digested PCR product with enzyme produced a digested produced 85, 48 and 26 bp in length while the PCR product from mutant sequence yield only two fragments of 85 and 74 bp. The DNA sequences of mutation of codons 12 and 13, which determined by automatic analyzer, were compared with the normal nucleotide sequences form Gene Bank ID: L00045.