CHAPTER III

RESULTS

3.1 GENOMIC DNA PREPARATION

The 82 genomic DNA samples were extracted from ovarian cancer tissue specimens. The yield of every sample was approximately range 450-1250 μg/ml and the purities were between 1.7 to 1.9.

3.2 AMPLIFIED CREATED RESTRICTION SITE METHOD WITH POLYMERASE CHAIN REACTION – RESRICTION FRAGMENT LENGTH POLYMORPHISM

3.2.1 The optimization condition of the amplified created restriction site method with polymerase chain reaction

The PCR process is widely employed in a tremendous variety of experimental applications to produce a clear single band DNA target sequences. Since no single set of conditions can be applied to all PCR amplifications, individual reaction component concentrations must be adjusted within suggested ranges for efficient amplification of specific targets. While there are a number of possible concentration parameters, logical titration of interrelated reaction components can be readily defined. In addition, the temperature optimal can often be determined within a few experiments.

A. The optimization condition of the amplified created restriction site method with polymerase chain reaction for codon 12 of K-ras gene

In this study, for the optimization of PCR reaction components, the annealing temperatures were varied from 56.0°C to 67.0°C (56.0°C, 59.0°C, 61.0°C, 64.0°C and 67.0°C) as shown in **Figure 23**. We found that at 60.0°C represented obviously annealing result without non-specific bands. The concentrations of deoxynucleoside 5'-triphosphates (dNTPs) were varied

from 50 to 200 μM (50, 100, 150, 200 and 250 μM), the optimization of dNTPs concentration is 200 μM in **Figure 24**. Moreover, the concentrations of magnesium chloride ($MgCl_2$) were titrated from 1.0 to 3.0 mM (1.0, 1.5, 2.0, 2.5 and 3.0 mM). The most efficient $MgCl_2$ concentration shown at 2.0 mM in **Figure 25**. Finally, the primers concentration were varied from 0.1 to 0.5 μM (0.1, 0.2, 0.3, 0.4 and 0.5 μM), the optimization of primer concentration is 0.3 μM. The result shown in **Figure 26**.

After the genomic DNA was amplified by using these appropriate conditions. The PCR product, which provided a clear single band at the expected size of 162 bp, as shown in the "third" lane of **Figure 26**. The 100 bp of DNA molecular weight markers were shown in the lane "M" of each figure.

B. The optimization condition of the amplified created restriction site method with polymerase chain reaction for codon 13 of K-*ras* gene

The annealing temperatures were varied from 51.0°C to 67.0°C (51.0°C, 54.0°C, 57.0°C, 60.0°C, 63.0°C and 67.0°C) as shown in **Figure 27**. We found that at 63.0°C represented obviously annealing result without non-specific bands. In this experiment, the concentrations of dNTPs were varied from 50 to 200 μM (50, 100, 150, 200 and 250 μM), the most efficient of dNTPs concentration is 200 μM in **Figure 28**. Moreover, the concentrations of $MgCl_2$ were varied from 1.0 to 3.0 mM (1.0, 1.5, 2.0, 2.5 and 3.0 mM), the optimization of $MgCl_2$ concentration shown at 2.0 mM in **Figure 29**. Finally, the primers were varied from 0.1 to 0.5 μM (0.1, 0.2, 0.3, 0.4 and 0.5 μM)), the appropriate concentration of primer is 0.2 μM in **Figure 30**.

After the genomic DNA was amplified by using these optimal conditions. The PCR product, which provided a clear single band at the expected size of 159 bp, as shown in the "second" lanes of **Figure 30**. The 100 bp of DNA molecular weight markers was shown in the lane "M" of each figure.

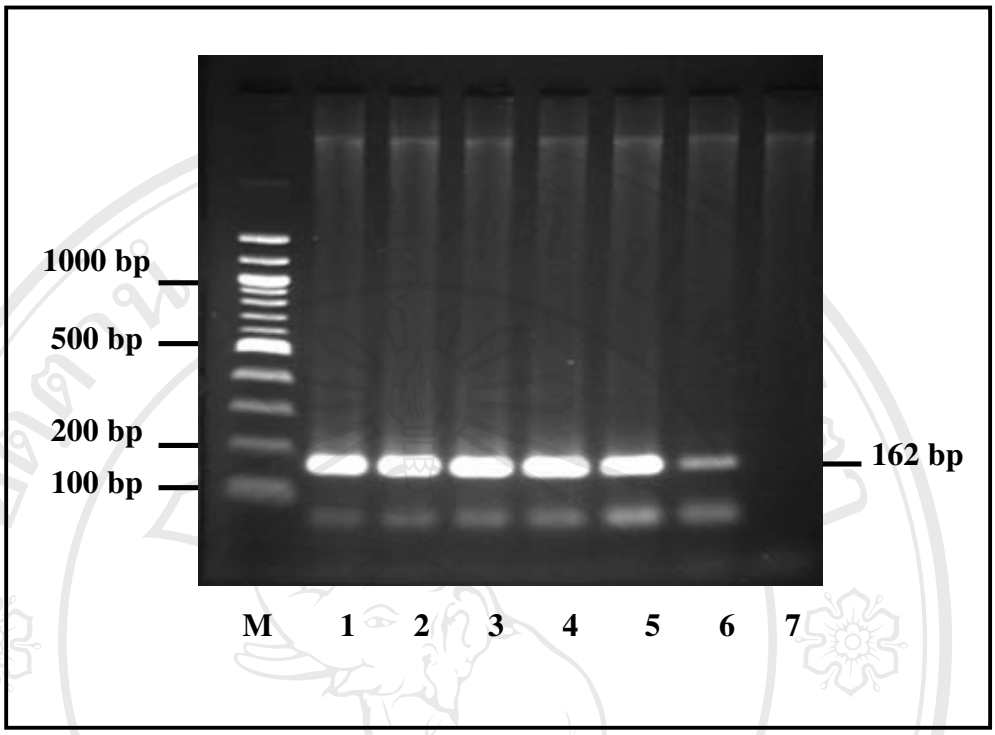

Figure 23 Agarose gel electrophoresis analysis of the optimization of the annealing temperatures for detection of codon 12 mutation of K-*ras* gene by ACRS-PCR.

The annealing temperatures were adjusted to provide the best result. The annealing temperatures were varied from 54.0°C to 72.0°C respectively. The PCR products were subjected to electrophoresis on 2%(w/v) agarose gel in 0.5X TBE buffer. Specifically sized of PCR product was obtained for the 162 bp.

Lane M = Standard DNA 100 bp molecular weight maker

Lane 1 = 54.0°C of annealing temperature

Lane 2 = 58.0°C of annealing temperature

Lane 3 = 60.0°C of annealing temperature

Lane 4 = 63.0°C of annealing temperature

Lane 5 = 66.0°C of annealing temperature

Lane 6 = 68.0°C of annealing temperature

Lane 7 = 72.0°C of annealing temperature

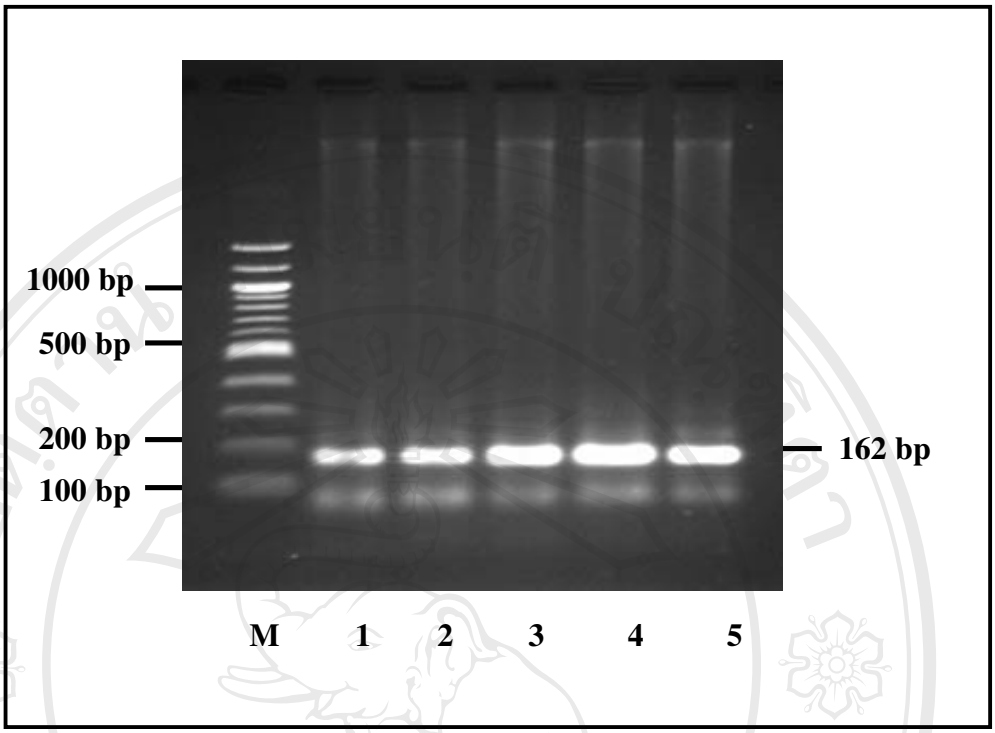

Figure 24 Agarose gel electrophoresis analysis of the optimization of the deoxynucleoside 5'-triphosphates concentrations for detection of codon 12 mutation of K-*ras* gene by ACRS-PCR.

The deoxynucleoside 5'-triphosphates (dNTPs) concentrations were adjusted to provide the best result. The dNTPs concentrations were varied from 50 μM to 250 μM respectively. The PCR products were subjected to electrophoresis on 2%(w/v) agarose gel in 0.5X buffer. Specifically sized of PCR product was obtained for the 162 bp.

Lane M = Standard DNA 100 bp molecular weight maker

Lane 1 = 50 μM of dNTPs concentration

Lane 2 = 100 μM of dNTPs concentration

Lane 3 = 150 μM of dNTPs concentration

Lane 4 = 200 μM of dNTPs concentration

Lane 5 = 250 μM of dNTPs concentration

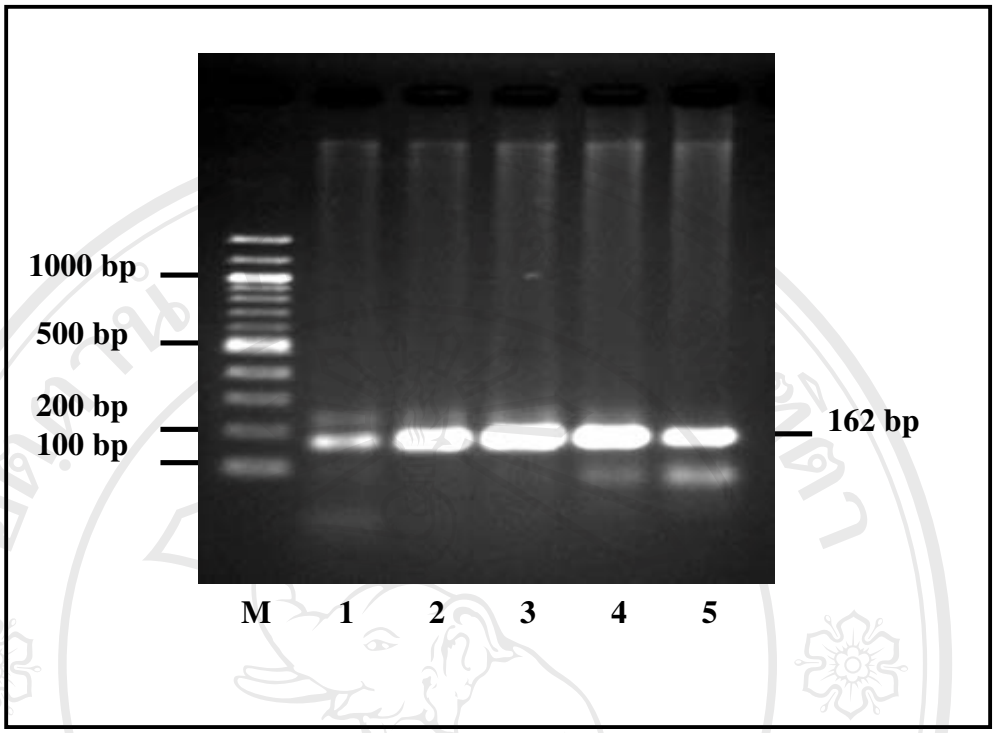

Figure 25 Agarose gel electrophoresis analysis of the optimization of the magnesium chloride concentrations for detection of codon 12 mutation of K-*ras* gene by ACRS -PCR.

The magnesium chloride ($MgCl_2$) concentrations were adjusted to provide the best result. The $MgCl_2$ concentrations were varied from 1.0 mM to 3.0 mM respectively. The PCR products were subjected to electrophoresis on 2%(w/v) agarose gel in 0.5X buffer. Specifically sized of PCR product was obtained for the 162 bp.

Lane M = Standard DNA 100 bp molecular weight maker

Lane 1 = 1.0 mM of $MgCl_2$ concentration

Lane 2 = 1.5 mM of $MgCl_2$ concentration

Lane 3 = 2.0 mM of $MgCl_2$ concentration

Lane 4 = 2.5 mM of $MgCl_2$ concentration

Lane 5 = 3.0 mM of $MgCl_2$ concentration

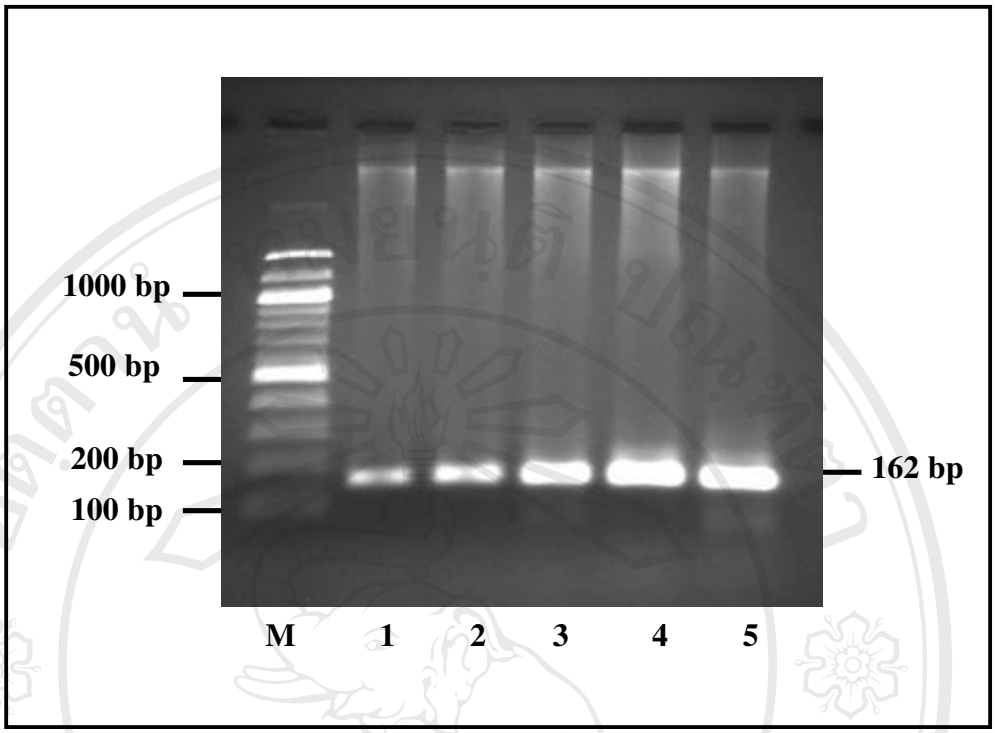

Figure 26 Agarose gel electrophoresis analysis of the optimization of the primer concentrations for detection of codon 12 mutation of K-*ras* gene by ACRS -PCR.

The primer concentrations were adjusted to provide the best result. The primer concentrations were varied from 100 μM to 500 μM respectively. The PCR products were subjected to electrophoresis on 2%(w/v) agarose gel in 0.5X buffer. Specifically sized of PCR product was obtained for the 162 bp.

Lane M = Standard DNA 100 bp molecular weight maker
Lane 1 = 0.1 μM of primer concentration
Lane 2 = 0.2 μM of primer concentration
Lane 3 = 0.3 μM of primer concentration
Lane 4 = 0.4 μM of primer concentration
Lane 5 = 0.5 μM of primer concentration

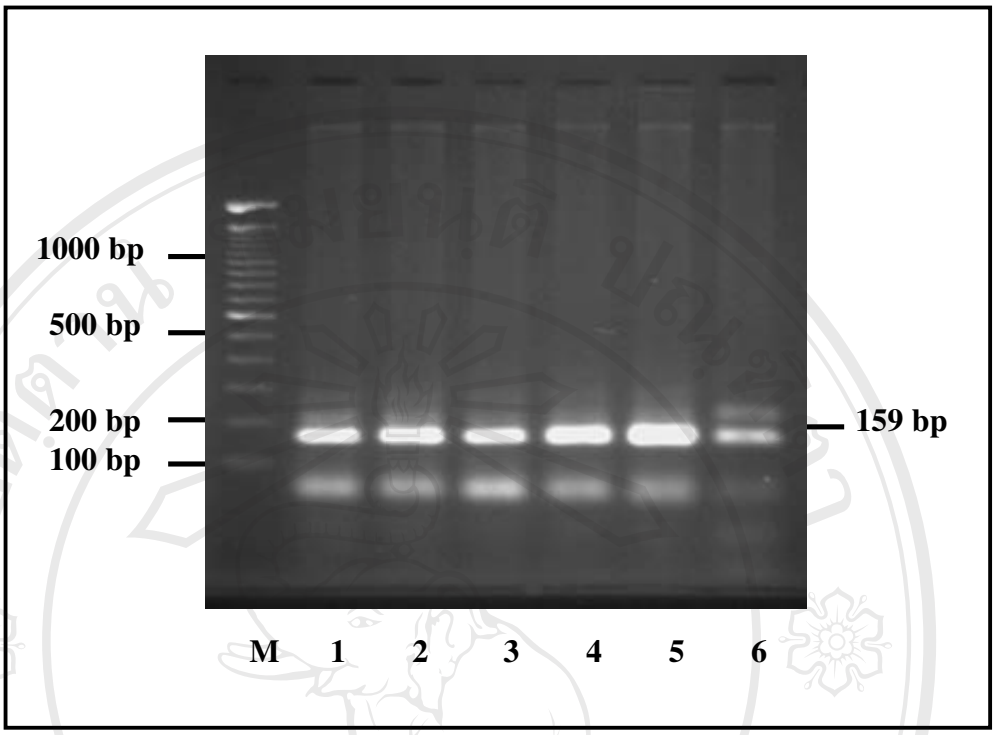

Figure 27 Agarose gel electrophoresis analysis of the optimization of the annealing temperatures for detection of codon 13 mutation of K-*ras* gene by ACRS-PCR.

The annealing temperatures were adjusted to provide the best result. The annealing temperatures were varied from 51.0°C to 67.0°C respectively. The PCR products were subjected to electrophoresis on 2%(w/v) agarose gel in 0.5X TBE buffer. Specifically sized of PCR product was obtained for the 159 bp.

Lane M = Standard DNA 100 bp molecular weight maker

Lane 1 = 51.0°C of annealing temperature

Lane 2 = 54.0°C of annealing temperature

Lane 3 = 57.0°C of annealing temperature

Lane 4 = 60.0°C of annealing temperature

Lane 5 = 63.0°C of annealing temperature

Lane 6 = 67.0°C of annealing temperature

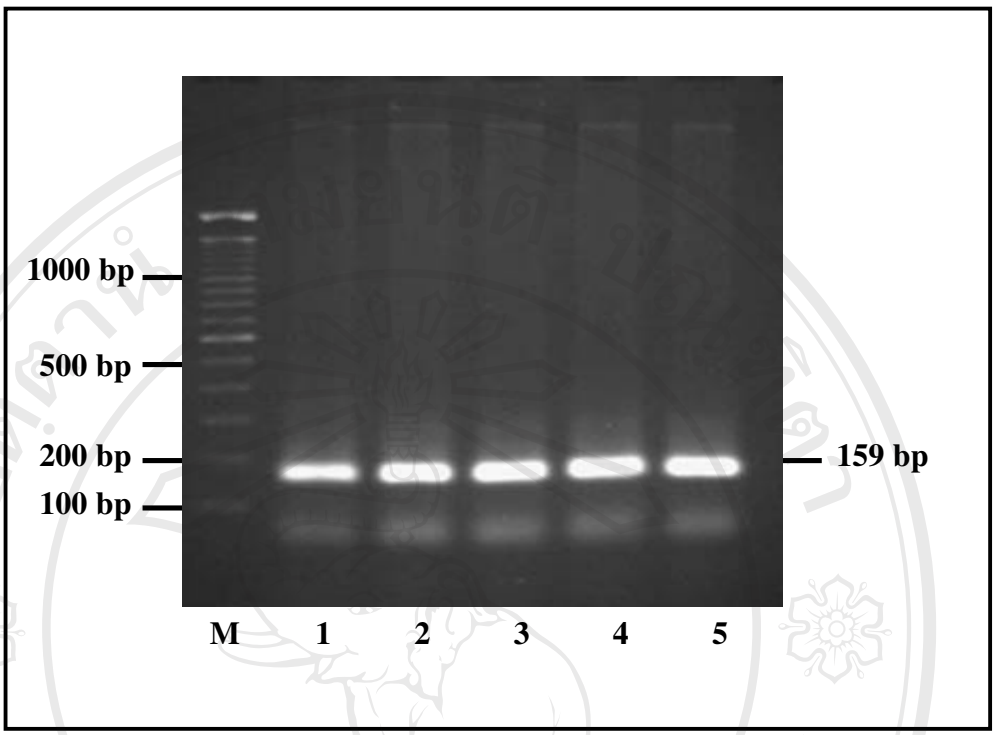

Figure 28 Agarose gel electrophoresis analysis of the optimization of the deoxynucleoside 5'-triphosphates concentrations for detection of codon 13 mutation of K–*ras* gene by ACRS -PCR.

The deoxynucleoside 5'-triphosphates (dNTPs) concentrations were adjusted to provide the best result. The dNTPs concentrations were varied from 50 μM to 250 μM respectively. The PCR products were subjected to electrophoresis on 2%(w/v) agarose gel in 0.5X buffer. Specifically sized of PCR product was obtained for the 159 bp.

Lane M = Standard DNA 100 bp molecular weight maker

Lane 1 = 50 μM of dNTPs concentration

Lane 2 = 100 μM of dNTPs concentration

Lane 3 = 150 μM of dNTPs concentration

Lane 4 = 200 μM of dNTPs concentration

Lane 5 = 250 μM of dNTPs concentration

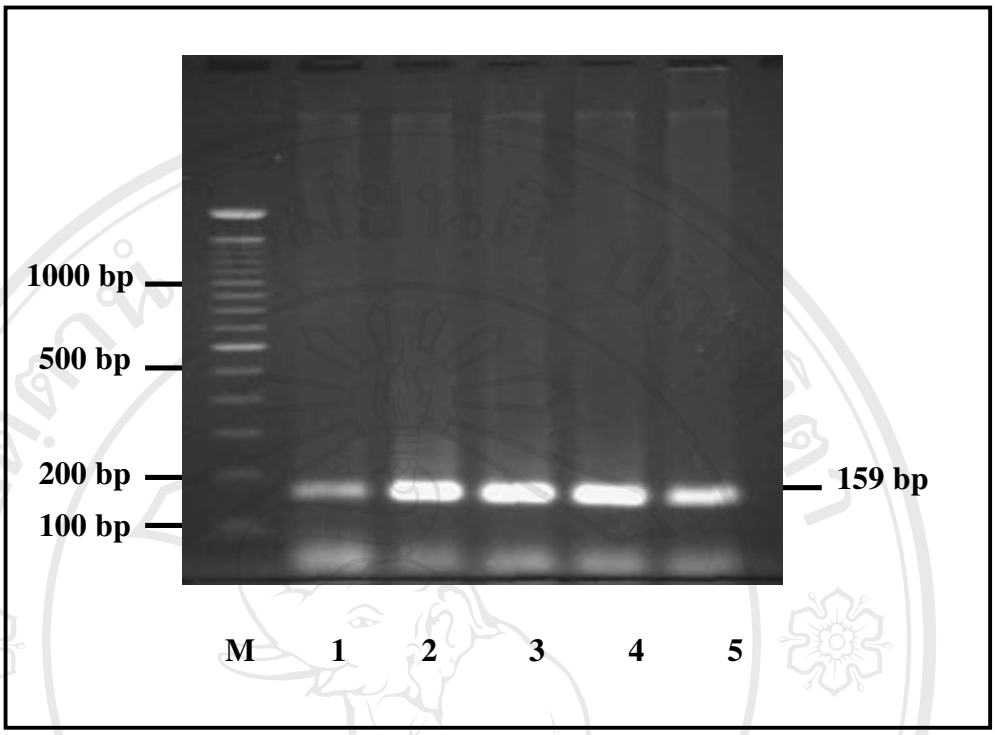

Figure 29 Agarose gel electrophoresis analysis of the optimization of the magnesium chloride concentrations for detection of codon 13 mutation of K-*ras* gene by ACRS-PCR.

The magnesium chloride ($MgCl_2$) concentrations were adjusted to provide the best result. The $MgCl_2$ concentrations were varied from 1.0 mM to 3.0 mM respectively. The PCR products were subjected to electrophoresis on 2%(w/v) agarose gel in 0.5X buffer. Specifically sized of PCR product was obtained for the 159 bp.

Lane M = Standard DNA 100 bp molecular weight maker

Lane 1 = 1.0 mM of $MgCl_2$ concentration

Lane 2 = 1.5 mM of $MgCl_2$ concentration

Lane 3 = 2.0 mM of $MgCl_2$ concentration

Lane 4 = 2.5 mM of $MgCl_2$ concentration

Lane 5 = 3.0 mM of $MgCl_2$ concentration

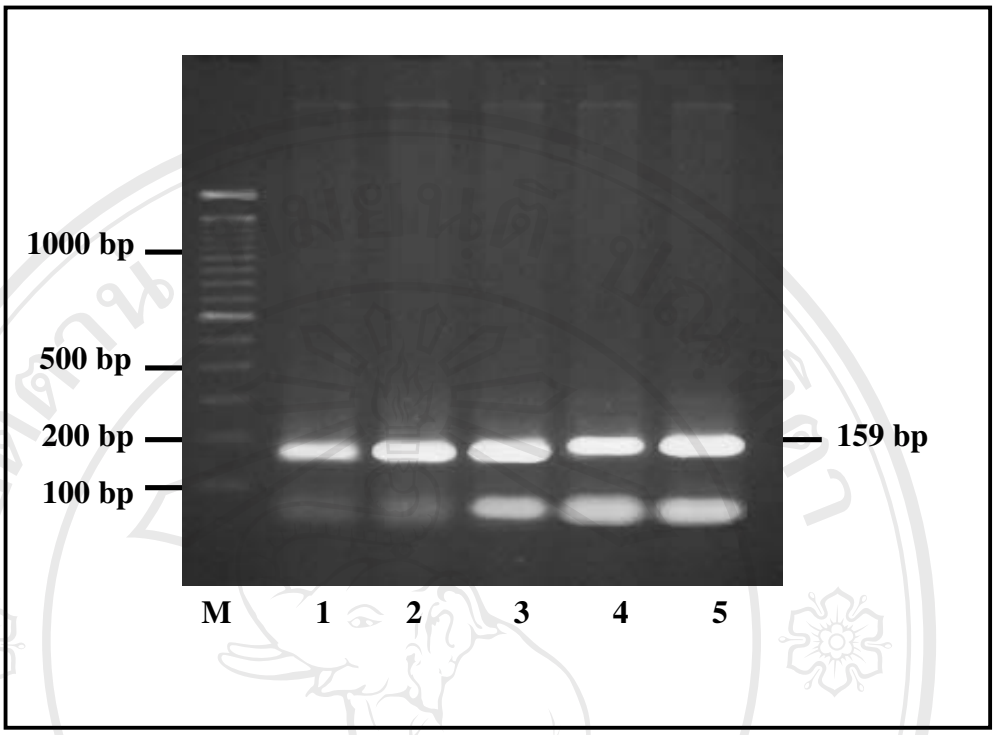

Figure 30 Agarose gel electrophoresis analysis of the optimization of the primer concentrations for detection of codon 13 mutation of K-*ras* gene by ACRS -PCR.

The primer concentrations were adjusted to provide the best result. The primer concentrations were varied from 100 μM to 500 μM respectively. The PCR products were subjected to electrophoresis on 2%(w/v) agarose gel in 0.5X buffer. Specifically sized of PCR product was obtained for the 159 bp.

Lane M = Standard DNA 100 bp molecular weight maker

Lane 1 = 0.1 μM of primer concentration

Lane 2 = 0.2 μM of primer concentration

Lane 3 = 0.3 μM of primer concentration

Lane 4 = 0.4 μM of primer concentration

Lane 5 = 0.5 μM of primer concentration

3.2.2 Restriction enzyme digestion

K-*ras* mutation at codon 12 was determined by restriction fragment length polymorphism (RFLP). The forward primer containing one base mismatch at 3' end amplified created a restriction site for "*Bst*NI" in the PCR product obtain from normal sequence at codon 12 (GGT), and produced a digested product of 133 bp and 29 bp in length, whereas the PCR product amplified from mutant sequence at codon 12 was absent this restriction site, and generated a 162 bp undigested product.

For detection of mutation at codon 13 of K-*ras* gene, the reverse primer containing one base mismatch at 5' end amplified created a second restriction site for "*Hea*III" in the PCR product obtain from normal sequence at codon 13 (CCG), and produced a digested product of 85, 48 and 26 bp in length while the PCR product amplified from mutant sequence yield only two fragments of 85 and 74 bp because of an internal *Hae*III site at nucleotide 85.

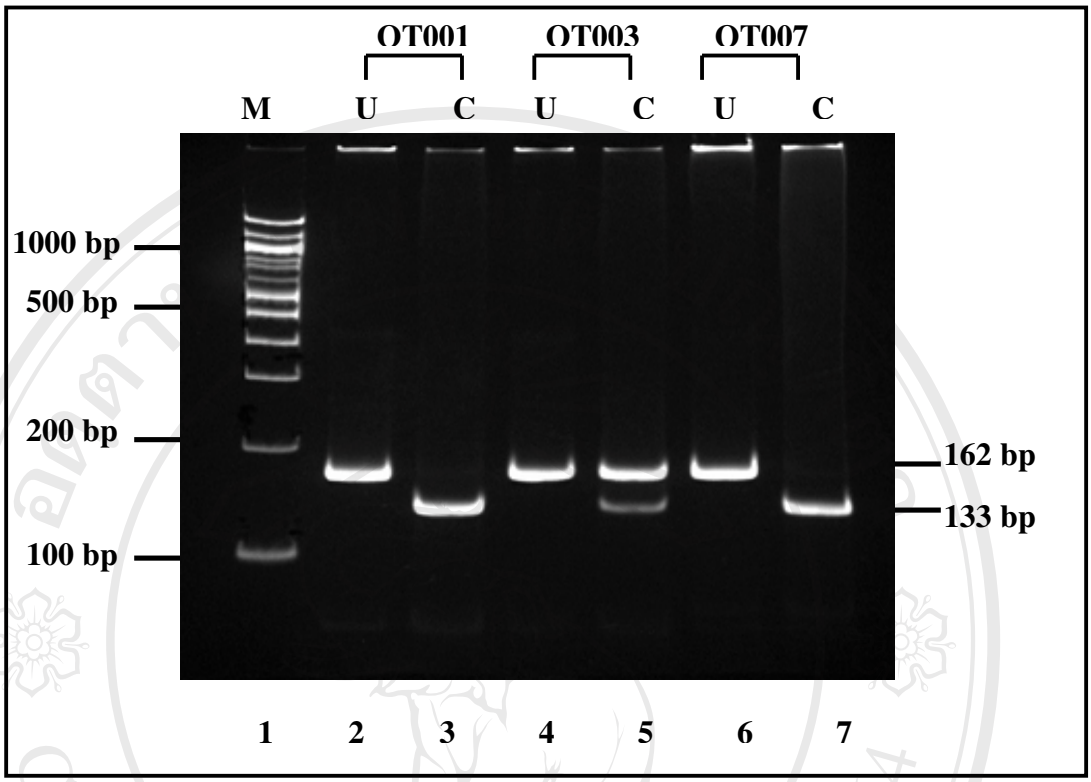

Figure 31 Polymerase chain reaction - restriction fragment length polymorphism analysis of point mutation at codon 12 of K-*ras* gene from ovarian cancer's tissues.

The PCR products before cutting with *BstN* I restriction enzyme are shown in lane "U" and after cutting are shown in lane "C". The standard DNA 100 bp ladder was shown in the lane "M". The number of each sample (OT) is shown at the above line. The PCR products were run on 12 % non-denaturing polyacrylamide gel in 1X TBE buffer.

Lane 1 = Standard DNA 100 bp molecular weight maker
Lane 2, 4, 6 = PCR products uncut with *BstN*I restriction enzyme size 162 bp
Lane 3, 7 = PCR product cut with *BstN*I restriction enzyme size 133 bp (Normal)
Lane 5 = PCR product cut with *BstN*I restriction enzyme size 162 and 133 bp (Mutant)

Note : PCR product fragment size 29 bp not visible

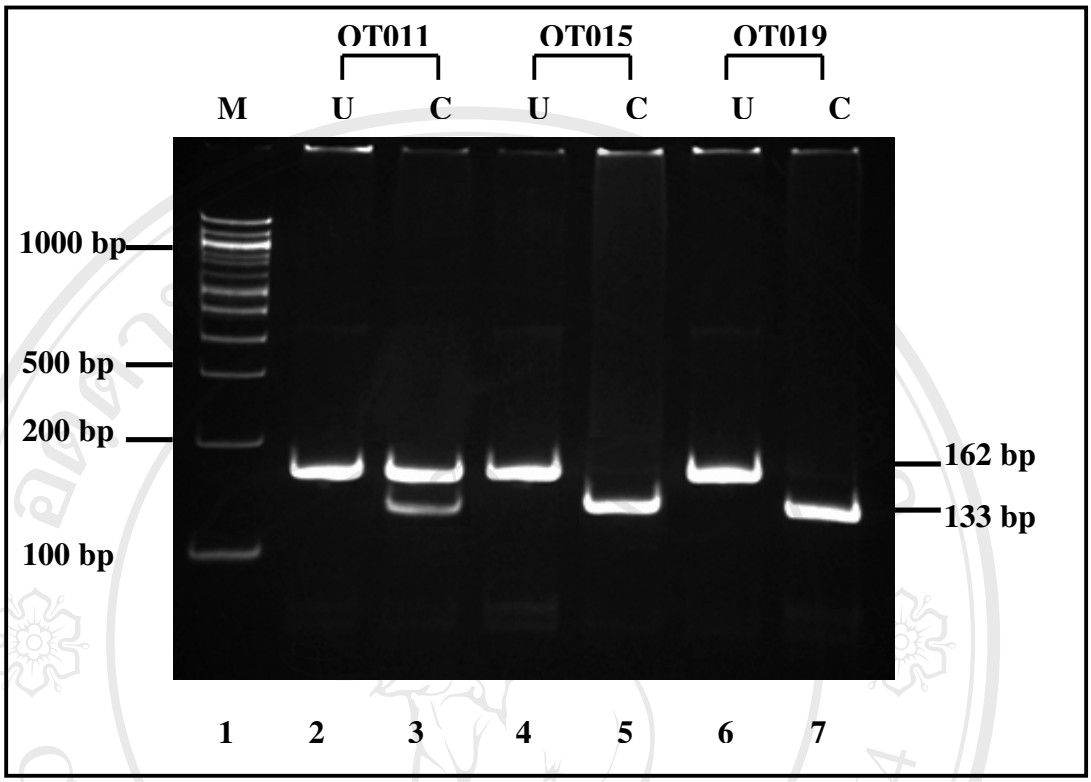

Figure 32 Polymerase chain reaction - restriction fragment length polymorphism analysis of point mutation at codon 12 of K-*ras* gene from ovarian cancer's tissues.

The PCR products before cutting with *Bst*NI restriction enzyme are shown in lane "U" and after cutting are shown in lane "C". The standard DNA 100 bp ladder was shown in the lane "M". The number of each sample (OT) is shown at the above line. The PCR products were run on 12 % non-denaturing polyacrylamide gel in 1X TBE buffer.

Lane 1 = Standard DNA 100 bp molecular weight maker

Lane 2, 4, 6 = PCR products uncut with *Bst*NI restriction enzyme size 162 bp

Lane 3 = PCR product cut with *Bst*NI restriction enzyme size 162 and 133 bp (Mutant)

Lane 5, 7 = PCR product cut with *Bst*NI restriction enzyme size 133 bp (Normal)

Note : PCR product fragment size 29 bp not visible

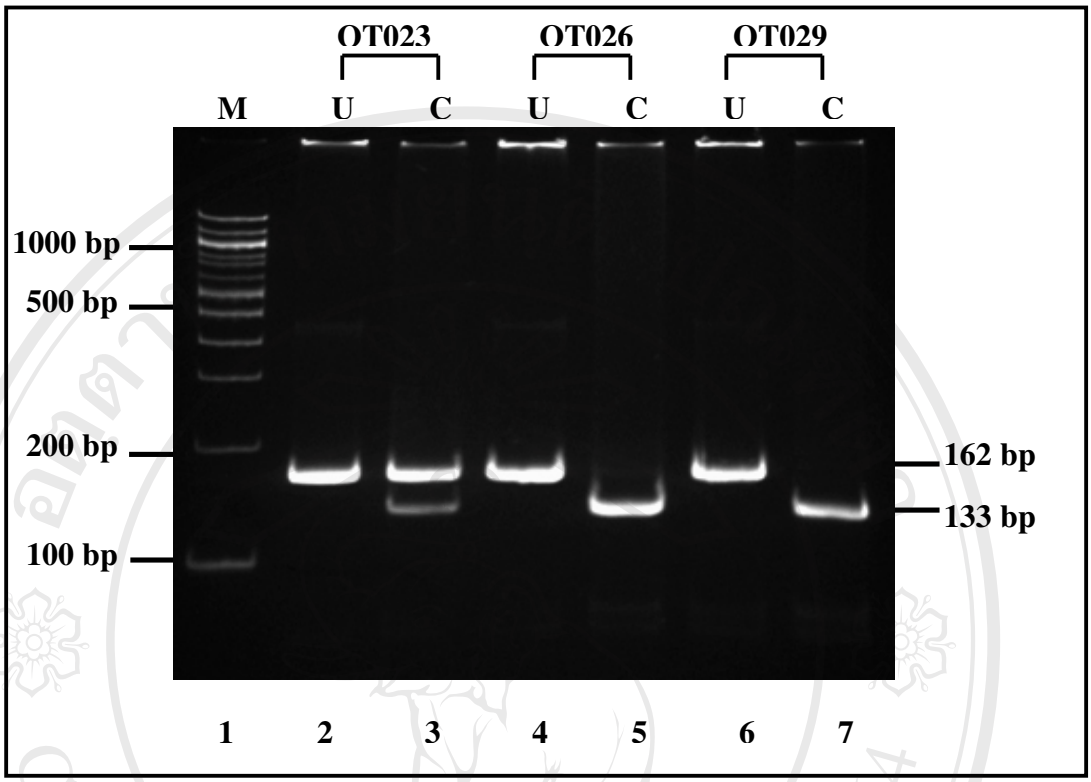

Figure 33 Polymerase chain reaction - restriction fragment length polymorphism analysis of point mutation at codon 12 of K-*ras* gene from ovarian cancer's tissues.

The PCR products before cutting with *BstN* I restriction enzyme are shown in lane "U" and after cutting are shown in lane "C". The standard DNA 100 bp ladder was shown in the lane "M". The number of each sample (OT) is shown at the above line. The PCR products were run on 12 % non-denaturing polyacrylamide gel in 1X TBE buffer.

Lane 1 = Standard DNA 100 bp molecular weight maker

Lane 2, 4, 6 = PCR products uncut with *Bst*NI restriction enzyme size 162 bp

Lane 3 = PCR product cut with *Bst*NI restriction enzyme size 162 and 133 bp (Mutant)

Lane 5, 7 = PCR product cut with *Bst*NI restriction enzyme size 133 bp (Normal)

Note : PCR product fragment size 29 bp not visible

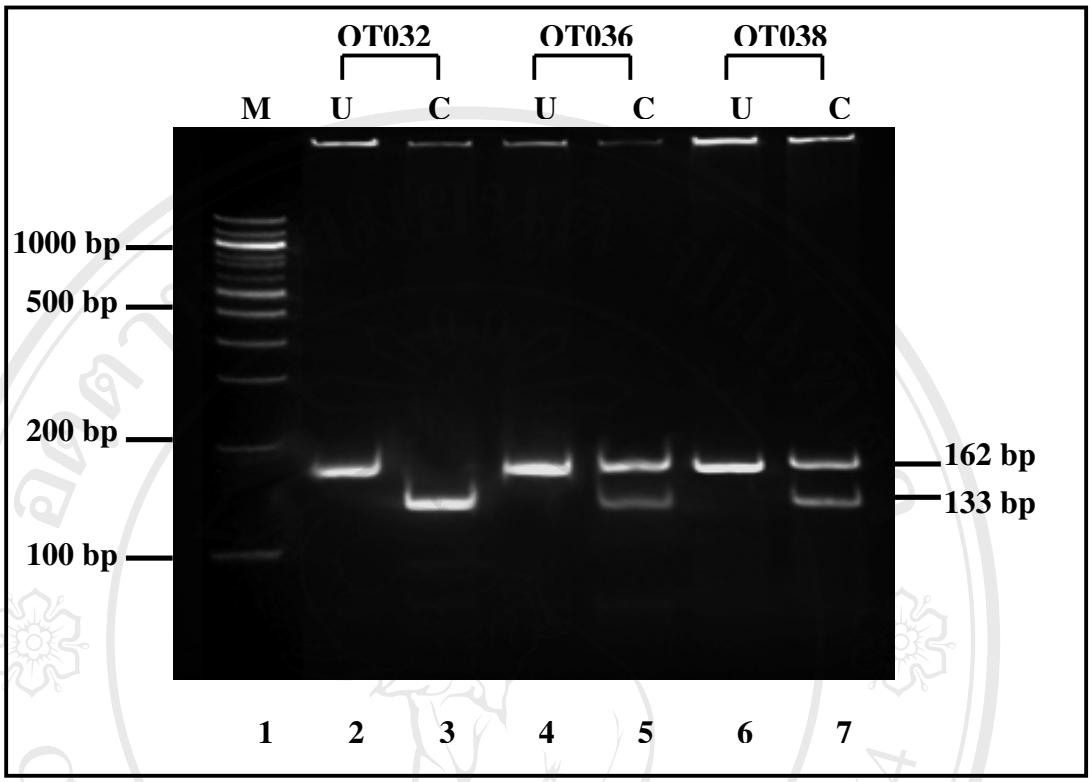

Figure 34 Polymerase chain reaction - restriction fragment length polymorphism analysis of point mutation at codon 12 of K-*ras* gene from ovarian cancer's tissues.

The PCR products before cutting with *Bst*NI restriction enzyme are shown in lane "U" and after cutting are shown in lane "C". The standard DNA 100 bp ladder was shown in the lane "M". The number of each sample (OT) is shown at the above line. The PCR products were run on 12 % non-denaturing polyacrylamide gel in 1X TBE buffer.

Lane 1 = Standard DNA 100 bp molecular weight maker
Lane 2, 4, 6 = PCR products uncut with *Bst*NI restriction enzyme size 162 bp
Lane 3 = PCR product cut with *Bst*NI restriction enzyme size 133 bp (Normal)
Lane 5, 7 = PCR product cut with *Bst*NI restriction enzyme size 162 and 133 bp (Mutant)

Note : PCR product fragment size 29 bp not visible

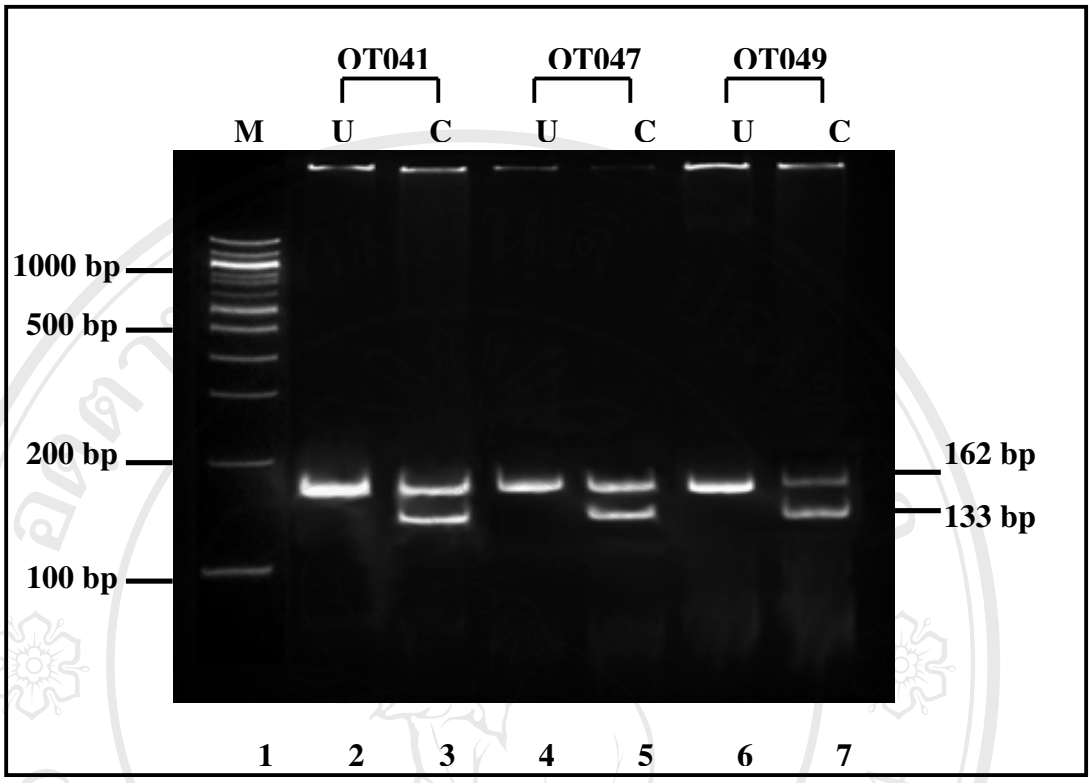

Figure 35 Polymerase chain reaction - restriction fragment length polymorphism analysis of point mutation at codon 12 of K-*ras* gene from ovarian cancer's tissues.

The PCR products before cutting with *Bst*NI restriction enzyme are shown in lane "U" and after cutting are shown in lane "C". The standard DNA 100 bp ladder was shown in the lane "M". The number of each sample (OT) is shown at the above line. The PCR products were run on 12 % non-denaturing polyacrylamide gel in 1X TBE buffer.

Lane 1 = Standard DNA 100 bp molecular weight maker

Lane 2, 4, 6 = PCR products uncut with *Bst*NI restriction enzyme size 162 bp

Lane 3, 5, 7 = PCR product cut with *Bst*NI restriction enzyme size 162 and 133 bp (Mutant)

Note : PCR product fragment size 29 bp not visible

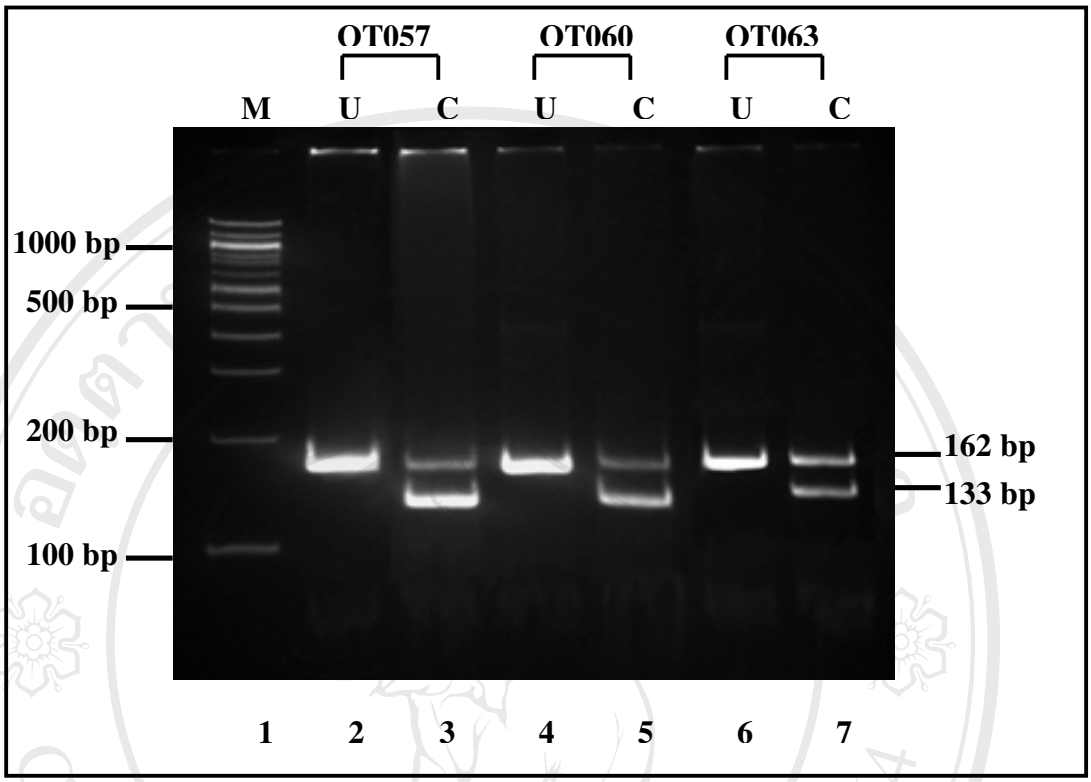

Figure 36 Polymerase chain reaction - restriction fragment length polymorphism analysis of point mutation at codon 12 of K-*ras* gene from ovarian cancer's tissues.

The PCR products before cutting with *Bst*NI restriction enzyme are shown in lane "U" and after cutting are shown in lane "C". The standard DNA 100 bp ladder was shown in the lane "M". The number of each sample (OT) is shown at the above line. The PCR products were run on 12 % non-denaturing polyacrylamide gel in 1X TBE buffer.

Lane 1 = Standard DNA 100 bp molecular weight maker

Lane 2, 4, 6 = PCR products uncut with *Bst*NI restriction enzyme size 162 bp

Lane 3, 5, 7 = PCR product cut with *Bst*NI restriction enzyme size 162 and 133 bp (Mutant)

Note : PCR product fragment size 29 bp not visible

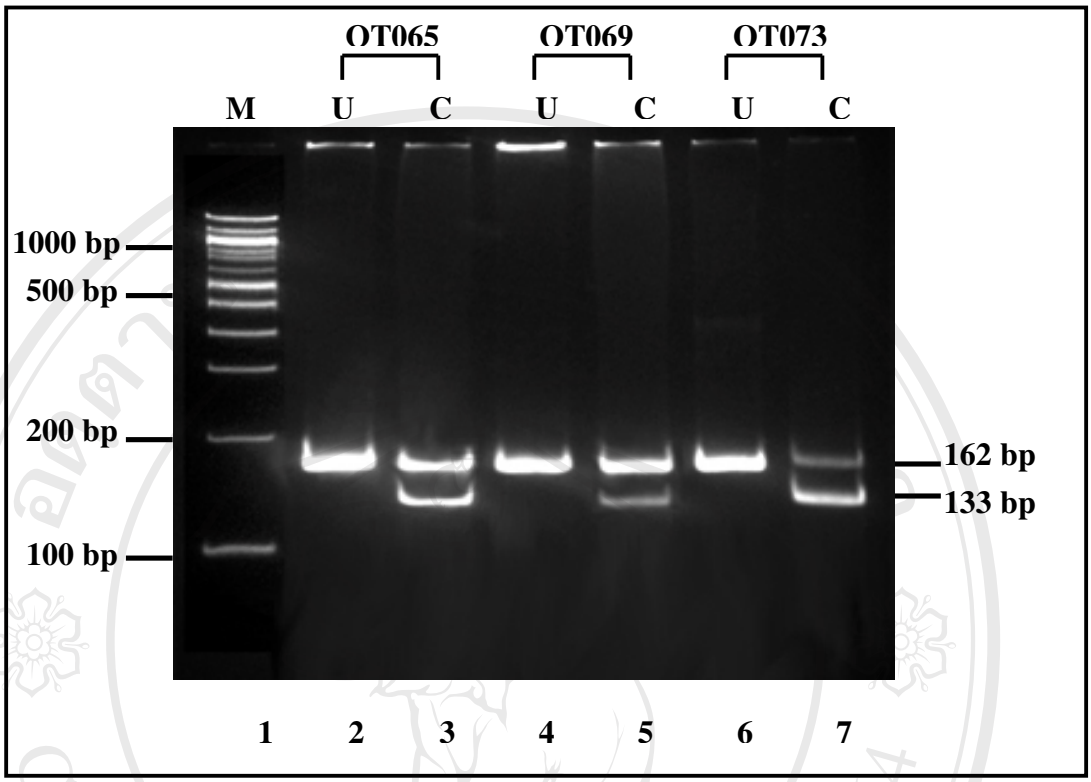

Figure 37 Polymerase chain reaction - restriction fragment length polymorphism analysis of point mutation at codon 12 of K-*ras* gene from ovarian cancer's tissues.

The PCR products before cutting with *Bst*NI restriction enzyme are shown in lane "U" and after cutting are shown in lane "C". The standard DNA 100 bp ladder was shown in the lane "M". The number of each sample (OT) is shown at the above line. The PCR products were run on 12 % non-denaturing polyacrylamide gel in 1X TBE buffer.

Lane 1 = Standard DNA 100 bp molecular weight maker
Lane 2, 4, 6 = PCR products uncut with *Bst*NI restriction enzyme size 162 bp
Lane 3, 5, 7 = PCR product cut with *Bst*NI restriction enzyme size 162 and 133 bp (Mutant)

Note : PCR product fragment size 29 bp not visible

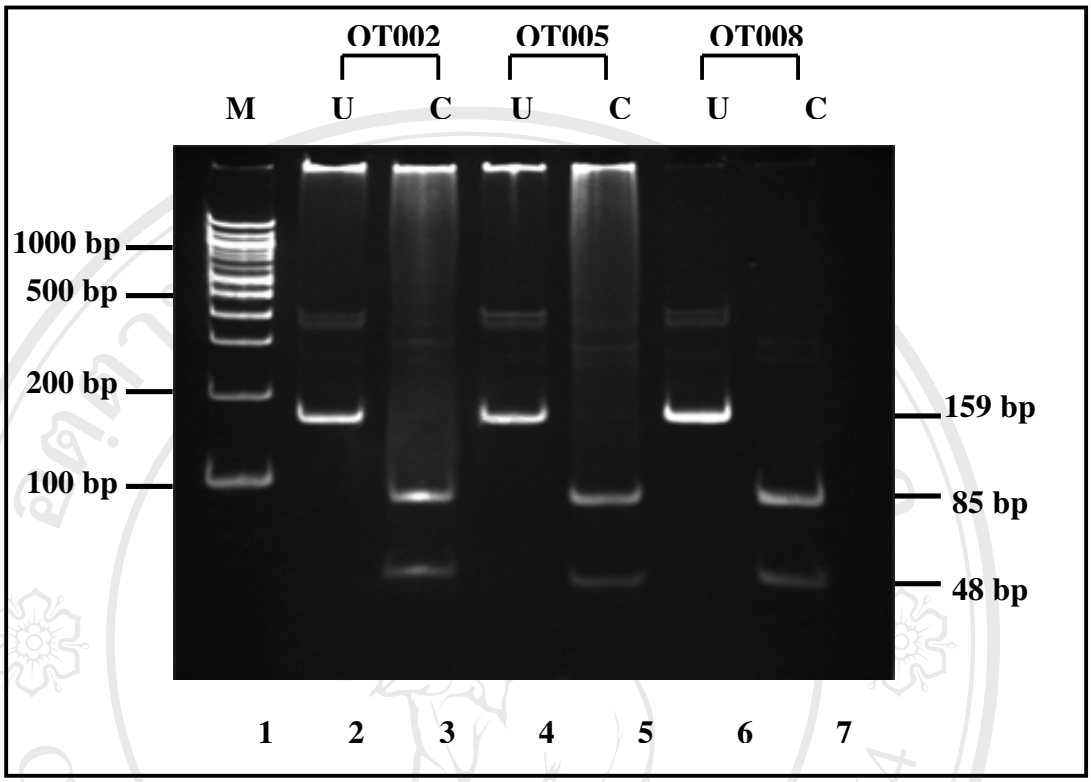

Figure 38 Polymerase chain reaction - restriction fragment length polymorphism analysis of point mutation at codon 13 of K-*ras* gene from ovarian cancer's tissues.

The PCR products before cutting with *Hea*III restriction enzyme are shown in lane "U" and after cutting are shown in lane "C". The standard DNA 100 bp ladder was shown in the lane "M". The number of each sample (OT) is shown at the above line. The PCR products were run on 12 % non-denaturing polyacrylamide gel in 1X TBE buffer.

Lane 1 = Standard DNA 100 bp molecular weight maker
Lane 2, 4, 6 = PCR products uncut with *Hea*III restriction enzyme size 159 bp
Lane 3, 5, 7 = PCR product cut with *Hea*III restriction enzyme size 85 and 48 bp (Normal)

Note : PCR product fragment size 26 bp not visible

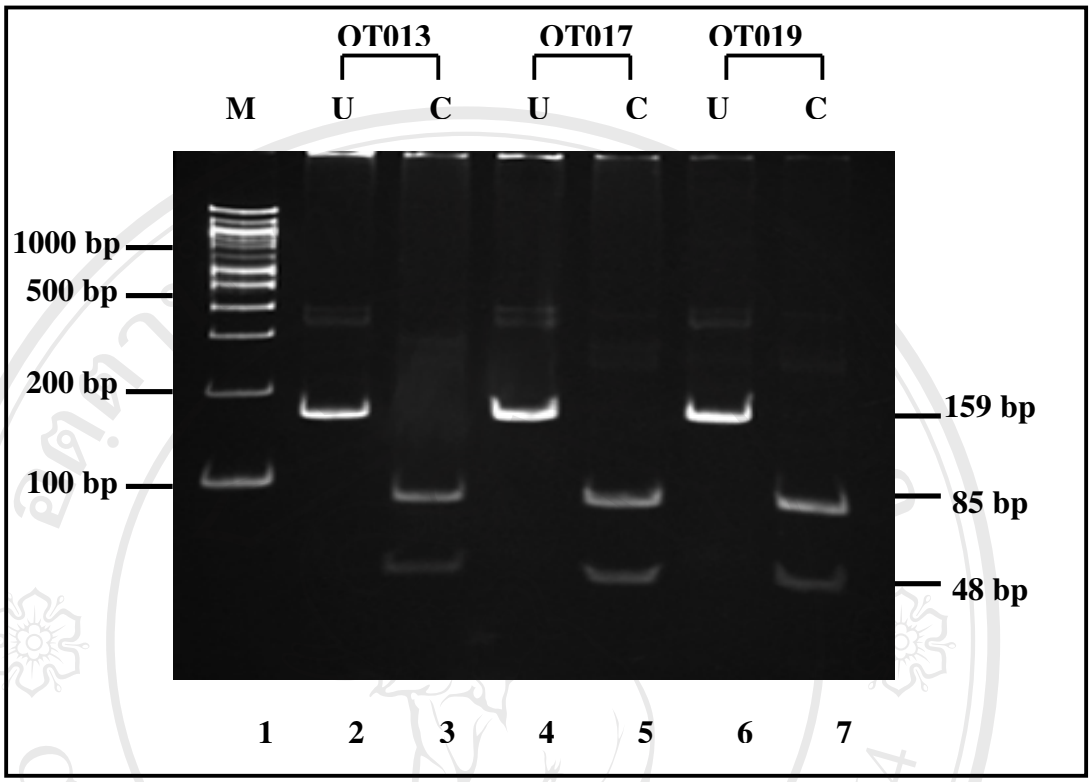

Figure 39 Polymerase chain reaction - restriction fragment length polymorphism analysis of point mutation at codon 13 of K-*ras* gene from ovarian cancer's tissues.

The PCR products before cutting with *Hea*III restriction enzyme are shown in lane "U" and after cutting are shown in lane "C". The standard DNA 100 bp ladder was shown in the lane "M". The number of each sample (OT) is shown at the above line. The PCR products were run on 12 % non-denaturing polyacrylamide gel in 1X TBE buffer.

Lane 1 = Standard DNA 100 bp molecular weight maker
Lane 2, 4, 6 = PCR products uncut with *Hea*III restriction enzyme size 159 bp
Lane 3, 5, 7 = PCR product cut with *Hea*III restriction enzyme size 85 and 48 bp (Normal)

Note : PCR product fragment size 26 bp not visible

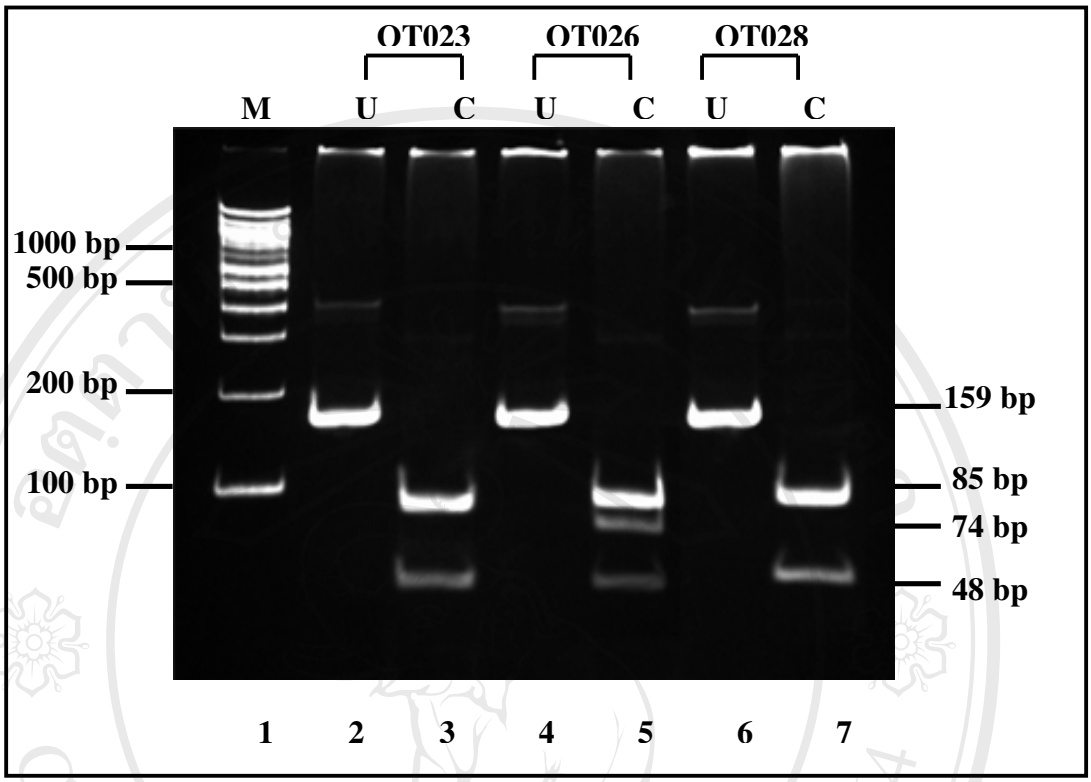

Figure 40 Polymerase chain reaction - restriction fragment length polymorphism analysis of point mutation at codon 13 of K-*ras* gene from ovarian cancer's tissues.

The PCR products before cutting with *Hea*III restriction enzyme are shown in lane "U" and after cutting are shown in lane "C". The standard DNA 100 bp ladder was shown in the lane "M". The number of each sample (OT) is shown at the above line. The PCR products were run on 12 % non-denaturing polyacrylamide gel in 1X TBE buffer.

Lane 1 = Standard DNA 100 bp molecular weight maker
Lane 2, 4, 6 = PCR products uncut with *Hea*III restriction enzyme size 159 bp
Lane 3, 7 = PCR product cut with *Hea*III restriction enzyme size 85 and 48 bp (Normal)
Lane 5 = PCR product cut with *Hea*III restriction enzyme size 85, 74 and 48 bp (Mutant)

Note : PCR product fragment size 26 bp not visible

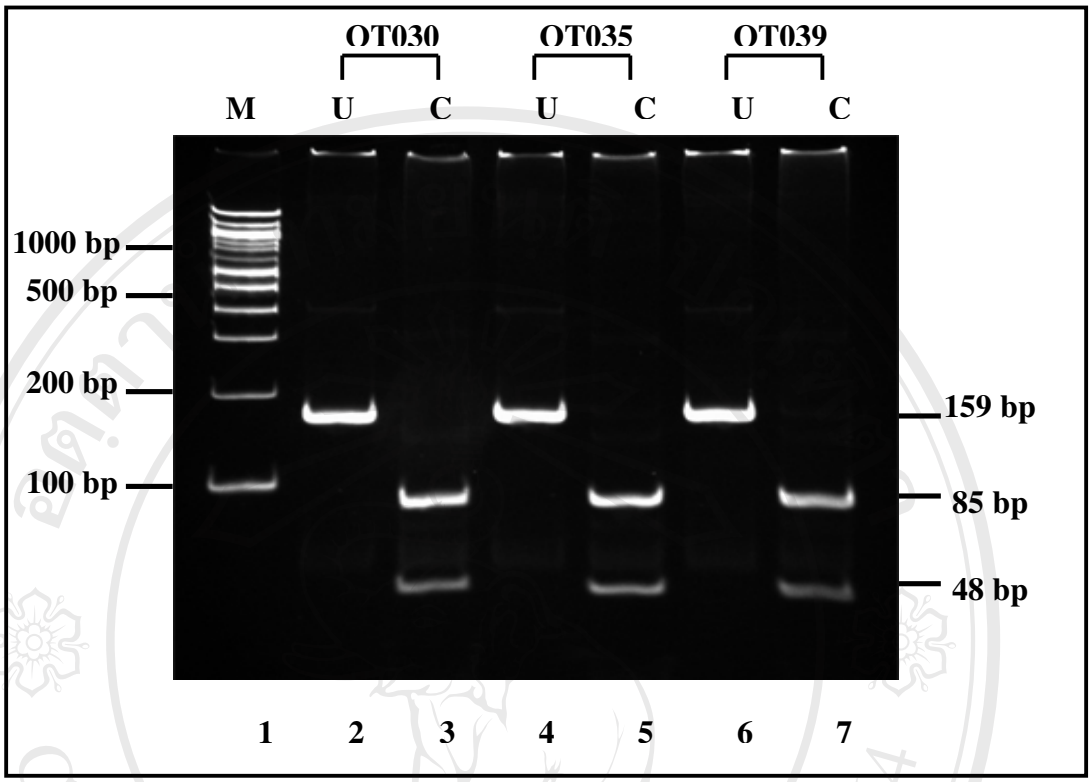

Figure 41 Polymerase chain reaction - restriction fragment length polymorphism analysis of point mutation at codon 13 of K-*ras* gene from ovarian cancer's tissues.

The PCR products before cutting with *Hea*III restriction enzyme are shown in lane "U" and after cutting are shown in lane "C". The standard DNA 100 bp ladder was shown in the lane "M". The number of each sample (OT) is shown at the above line. The PCR products were run on 12 % non-denaturing polyacrylamide gel in 1X TBE buffer.

Lane 1 = Standard DNA 100 bp molecular weight maker

Lane 2, 4, 6 = PCR products uncut with *Hea*III restriction enzyme size 159 bp

Lane 3, 5, 7 = PCR product cut with *Hea*III restriction enzyme size 85 and 48 bp (Normal)

Note : PCR product fragment size 26 bp not visible

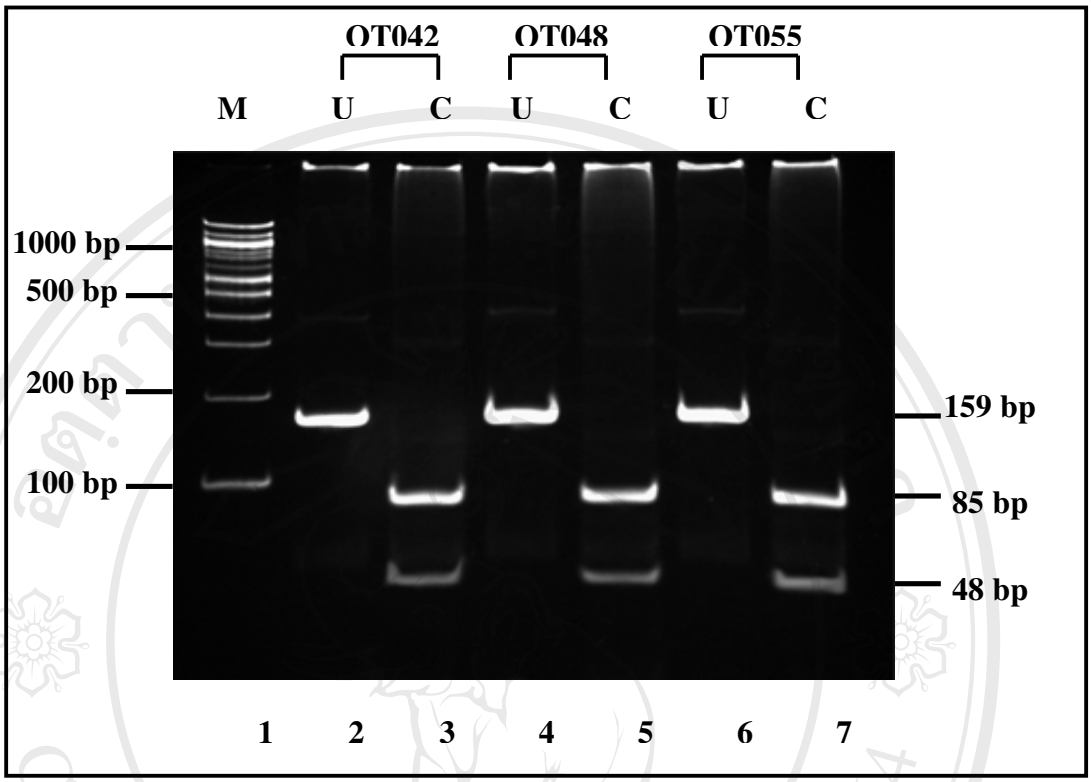

Figure 42 Polymerase chain reaction - restriction fragment length polymorphism analysis of point mutation at codon 13 of K-*ras* gene from ovarian cancer's tissues.

The PCR products before cutting with *Hea*III restriction enzyme are shown in lane "U" and after cutting are shown in lane "C". The standard DNA 100 bp ladder was shown in the lane "M". The number of each sample (OT) is shown at the above line. The PCR products were run on 12 % non-denaturing polyacrylamide gel in 1X TBE buffer.

Lane 1 = Standard DNA 100 bp molecular weight maker
Lane 2, 4, 6 = PCR products uncut with *Hea*III restriction enzyme size 159 bp
Lane 3, 5, 7 = PCR product cut with *Hea*III restriction enzyme size 85 and 48 bp (Normal)

Note : PCR product fragment size 26 bp not visible

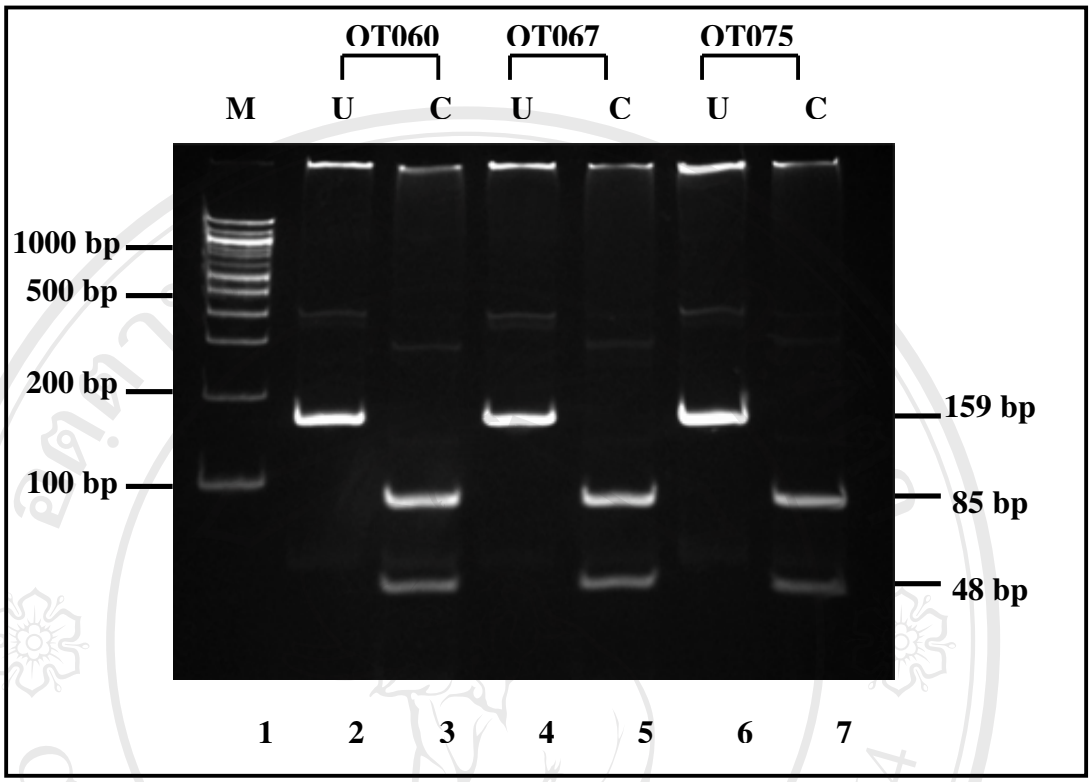

Figure 43 Polymerase chain reaction - restriction fragment length polymorphism analysis of point mutation at codon 13 of K-*ras* gene from ovarian cancer's tissues.

The PCR products before cutting with *Hea*III restriction enzyme are shown in lane "U" and after cutting are shown in lane "C". The standard DNA 100 bp ladder was shown in the lane "M". The number of each sample (OT) is shown at the above line. The PCR products were run on 12 % non-denaturing polyacrylamide gel in 1X TBE buffer.

Lane 1 = Standard DNA 100 bp molecular weight maker
Lane 2, 4, 6 = PCR products uncut with *Hea*III restriction enzyme size 159 bp
Lane 3, 5, 7 = PCR product cut with *Hea*III restriction enzyme size 85 and 48 bp (Normal)

Note : PCR product fragment size 26 bp not visible

3.2.3 The results from the analyzation of mutations at codon 12 and codon 13 on exon I of K-*ras* gene by amplified created restriction site method with polymerase chain reaction - restriction fragment length polymorphism

In this study, DNA amplified from each of the 82 ovarian cancer specimens was analyzed for mutations at codon 12 and codon 13 on exon I of the K-*ras* gene by ACRS with PCR- RFLP. The mutation at codon 12 on exon I of the K-*ras* gene was detected in 20% (14/82) of ovarian cancer specimens (cases OT003, OT011, OT023, OT036, OT038, OT041, OT047, OT049, OT057, OT060, OT063, OT065, OT069, and OT073) **(Table 2)**. One mutation was detected in codons 13 on exon I of the K-*ras* gene in ovarian cancer specimen (1% (1/82)) (cases OT026) (**Table 2**).

Table 2 The type of K-*ras* gene mutation at codon 12 and codon 13 by ACRS with PCR-RFLP in 82 Thai ovarian cancer specimens.

Case numbers	DNA extracted from tissue	The type of K-*ras* gene mutation by ACRS with PCR-RFLP			
		codon 12		codon 13	
		PCR products (bp)	Type	PCR products (bp)	Type
1	OT 001	133	Normal	85, 48	Normal
2	OT 002	133	Normal	85, 48	Normal
3	OT 003	162, 133	Mutant	85, 48	Normal
4	OT 004	133	Normal	85, 48	Normal
5	OT 005	133	Normal	85, 48	Normal
6	OT 006	133	Normal	85, 48	Normal
7	OT 007	133	Normal	85, 48	Normal
8	OT 008	133	Normal	85, 48	Normal
9	OT 009	133	Normal	85, 48	Normal
10	OT 010	133	Normal	85, 48	Normal
11	OT 011	162, 133	Mutant	85, 48	Normal

Table 2 (continued) The type of K-*ras* gene mutation at codon 12 and codon 13 by ACRS with PCR-RFLP in 82 Thai ovarian cancer specimens.

Case numbers	DNA extracted from tissue	The type of K-*ras* gene mutation by ACRS with PCR-RFLP			
		codon 12		codon 13	
		PCR products (bp)	Type	PCR products (bp)	Type
12	OT 012	133	Normal	85, 48	Normal
13	OT 013	133	Normal	85, 48	Normal
14	OT 014	133	Normal	85, 48	Normal
15	OT 015	133	Normal	85, 48	Normal
16	OT 016	133	Normal	85, 48	Normal
17	OT 017	133	Normal	85, 48	Normal
18	OT 018	133	Normal	85, 48	Normal
19	OT 019	133	Normal	85, 48	Normal
20	OT 020	133	Normal	85, 48	Normal
21	OT 021	133	Normal	85, 48	Normal
22	OT 022	133	Normal	85, 48	Normal
23	OT 023	162, 133	Mutant	85, 48	Normal
24	OT 024	133	Normal	85, 48	Normal
25	OT 025	133	Normal	85, 48	Normal
26	OT 026	133	Normal	74, 85, 48	Mutant
27	OT 027	133	Normal	85, 48	Normal
28	OT 028	133	Normal	85, 48	Normal
29	OT 029	133	Normal	85, 48	Normal
30	OT 030	133	Normal	85, 48	Normal
31	OT 031	133	Normal	85, 48	Normal

Table 2 (continued) The type of K-*ras* gene mutation at codon 12 and codon 13 by ACRS with PCR-RFLP in 82 Thai ovarian cancer specimens.

Case numbers	DNA extracted from tissue	The type of K-*ras* gene mutation by ACRS with PCR-RFLP			
		codon 12		codon 13	
		PCR products (bp)	Type	PCR products (bp)	Type
32	OT 032	133	Normal	85, 48	Normal
33	OT 033	133	Normal	85, 48	Normal
34	OT 034	133	Normal	85, 48	Normal
35	OT 035	133	Normal	85, 48	Normal
36	OT 036	162, 133	Mutant	85, 48	Normal
37	OT 037	133	Normal	85, 48	Normal
38	OT 038	162, 133	Mutant	85, 48	Normal
39	OT 039	133	Normal	85, 48	Normal
40	OT 040	133	Normal	85, 48	Normal
41	OT 041	162, 133	Mutant	85, 48	Normal
42	OT 042	133	Normal	85, 48	Normal
43	OT 043	133	Normal	85, 48	Normal
44	OT 044	133	Normal	85, 48	Normal
45	OT 045	133	Normal	85, 48	Normal
46	OT 046	133	Normal	85, 48	Normal
47	OT 047	162, 133	Mutant	85, 48	Normal
48	OT 048	133	Normal	85, 48	Normal
49	OT 049	133	Mutant	85, 48	Normal
50	OT 050	133	Normal	85, 48	Normal
51	OT 051	133	Normal	85, 48	Normal

Table 2 (continued) The type of K-*ras* gene mutation at codon 12 and codon 13 by ACRS with PCR-RFLP in 82 Thai ovarian cancer specimens.

Case numbers	DNA extracted from tissue	The type of K-*ras* gene mutation by ACRS with PCR-RFLP			
		codon 12		codon 13	
		PCR products (bp)	Type	PCR products (bp)	Type
52	OT 052	133	Normal	85, 48	Normal
53	OT 053	133	Normal	85, 48	Normal
54	OT 054	133	Normal	85, 48	Normal
55	OT 055	133	Normal	85, 48	Normal
56	OT 056	133	Normal	85, 48	Normal
57	OT 057	162, 133	Mutant	85, 48	Normal
58	OT 058	133	Normal	85, 48	Normal
59	OT 059	133	Normal	85, 48	Normal
60	OT 060	162, 133	Mutant	85, 48	Normal
61	OT 061	133	Normal	85, 48	Normal
62	OT 062	133	Normal	85, 48	Normal
63	OT 063	162, 133	Mutant	85, 48	Normal
64	OT 064	133	Normal	85, 48	Normal
65	OT 065	162, 133	Mutant	85, 48	Normal
66	OT 066	133	Normal	85, 48	Normal
67	OT 067	133	Normal	85, 48	Normal
68	OT 068	133	Normal	85, 48	Normal
69	OT 069	162, 133	Mutant	85, 48	Normal
70	OT 070	133	Normal	85, 48	Normal
71	OT 071	133	Normal	85, 48	Normal

Table 2 (continued) The type of K-*ras* gene mutation at codon 12 and codon 13 by ACRS with PCR-RFLP in 82 Thai ovarian cancer specimens.

Case numbers	DNA extracted from tissue	The type of K -*ras* gene mutation by ACRS with PCR-RFLP			
		codon 12		codon 13	
		PCR products (bp)	Type	PCR products (bp)	Type
72	OT 072	133	Normal	85, 48	Normal
73	OT 073	162, 133	Mutant	85, 48	Normal
74	OT 074	133	Normal	85, 48	Normal
75	OT 075	133	Normal	85, 48	Normal
76	OT 076	133	Normal	85, 48	Normal
77	OT 077	133	Normal	85, 48	Normal
78	OT 078	133	Normal	85, 48	Normal
79	OT 079	133	Normal	85, 48	Normal
80	OT 080	133	Normal	85, 48	Normal
81	OT 081	133	Normal	85, 48	Normal
82	OT 082	133	Normal	85, 48	Normal

Table 3 The summarization of the mutations at codon 12 and codon 13 of K-*ras* gene by ACRS with PCR-RFLP in 82 Thai ovarian cancer specimens.

Tumor histology	Total number of cases	Number of cases with K-*ras* mutation (%)
Epithelial tumors		
Mucinous cystadenoma	2	0 (0%)
Mucinous tumors of LMP	16	8 (50%)
Mucinous adenocarcinomas	4	2 (50%)
Serous tumors of LMP	5	2 (40%)
Serous adenocarcinomas	13	0 (0%)
Clear cell adenocarcinomas	6	0 (0%)
Endometrioid tumors of LMP	1	1 (100%)
Endometrioid adenocarcimas	10	0 (0%)
Mixed epithelial tumor	2	0 (0%)
Mixed epithelial tumors of LMP	1	1 (100%)
Sex cord-stromal tumors		
Granulosa cell tumor	4	0 (0%)
Unclassified sex-cord tumor	1	0 (0%)
Germ cell tumors		
Immature teratomas	3	0 (0%)
Squamous cell carcinoma arising in teratoma	1	0 (0%)
Solid mature teratoma	1	0 (0%)
Metastatic tumor		
Matastatic adenocarcinoma	7	1 (14%)
Metastatic SCC from Cevix	1	0 (0%)
Matastatic signetring cell carcinoma	3	0 (0%)
Matastatic ductal carcinoma	1	0 (0%)

3.3 DNA SEQUENCING

The ovarian cancer specimens that exhibited the mutation at codon 12 and codon 13 on exon I of the K-*ras* gene by the amplified created restriction site method with PCR-RFLP analysis were further characterized the specific base substitution in both codons. DNA sequences of the first exon of the K-*ras* gene were amplified by PCR and sequenced by dideoxynucleotides chain termination method.

3.3.1 The optimization condition of PCR for sequenced exon I of K-*ras* gene

In this study, for the optimization of PCR reaction components, the annealing temperatures were varied from 56.0°C to 69.0°C (56.0°C, 59.0°C, 61.0°C, 64.0°C, 67.0°C and 69.0°C) as shown in **Figure 44**. We found that at 63.0°C represented obviously annealing results with high efficient. In this experiment, the concentration of $MgCl_2$ was varied from 1.0 to 3.0 mM (1.0, 1.5, 2.0, 2.5 and 3.0 mM), the optimal concentration of $MgCl_2$ shown at 2.5 mM in **Figure 45**.

After the genomic DNA was amplified by using appropriate conditions. The PCR products, which provided a clear single band at the expected size of 260 bp, as shown in the "forth" lane of **Figure 45**.

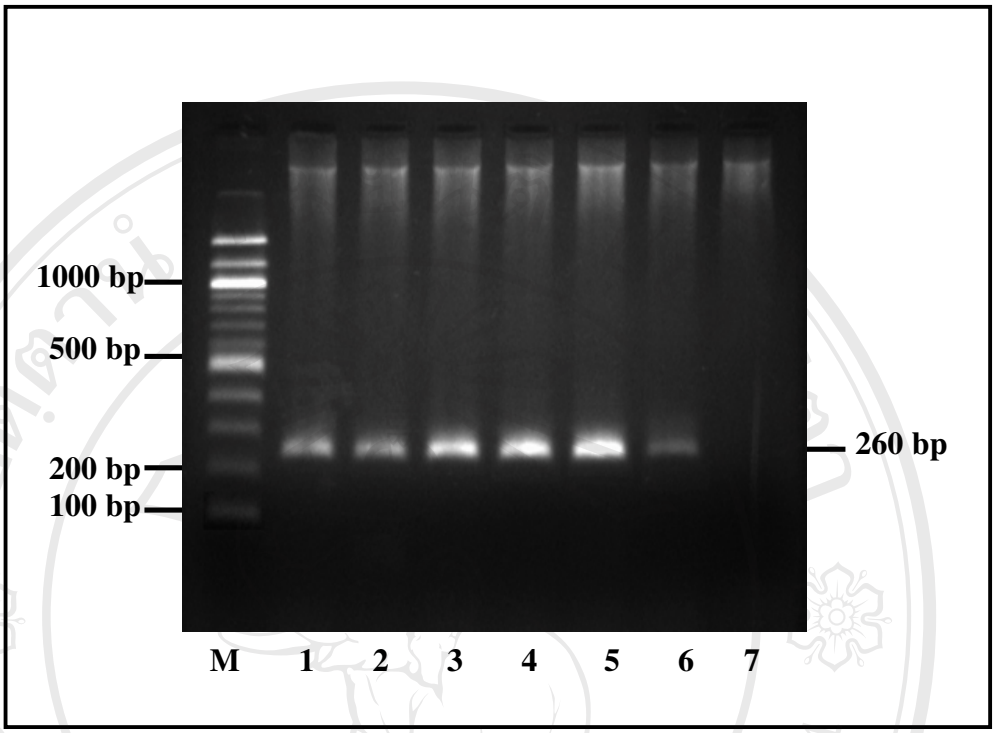

Figure 44 Agarose gel electrophoresis analysis of the optimization of the annealing temperatures for sequenced exon I of K-*ras* gene.

The annealing temperatures were adjusted to provide the best result. The annealing temperatures were varied from 51.0°C to 69.0°C respectively. The PCR products were subjected to electrophoresis on 2%(w/v) agarose gel in 0.5X TBE buffer. Specifically sized of PCR product was obtained for the 260 bp.

Lane M = Standard DNA 100 bp molecular weight maker

Lane 1 = 51.0°C of annealing temperature

Lane 2 = 54.0°C of annealing temperature

Lane 3 = 57.0°C of annealing temperature

Lane 4 = 60.0°C of annealing temperature

Lane 5 = 63.0°C of annealing temperature

Lane 6 = 67.0°C of annealing temperature

Lane 7 = 69.0°C of annealing temperature

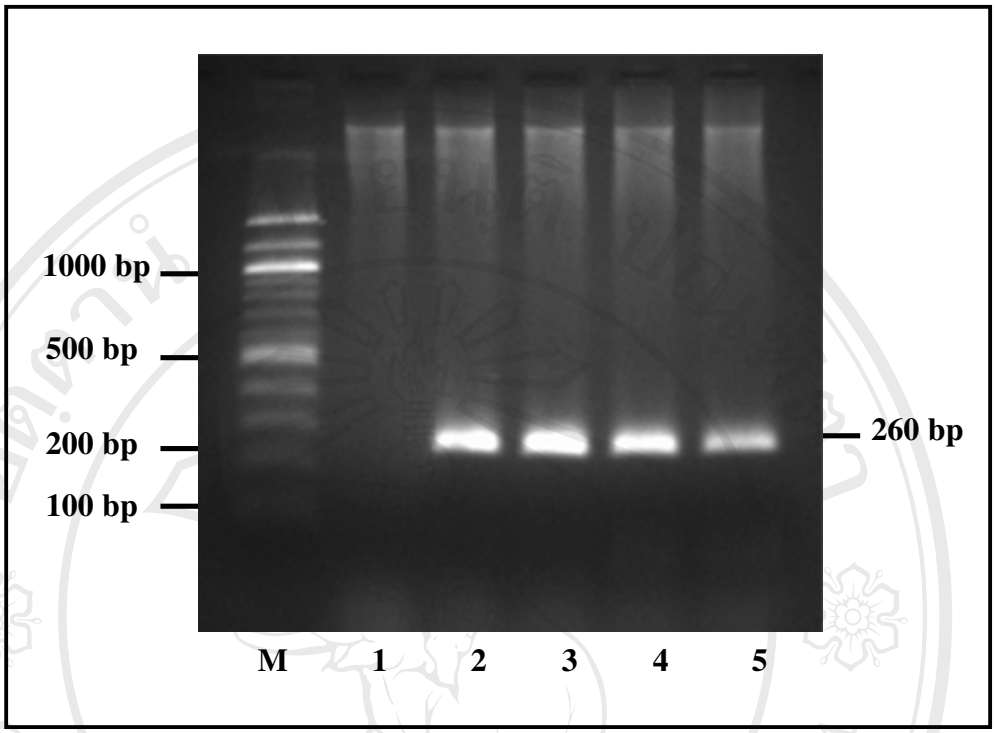

Figure 45 Agarose gel electrophoresis analysis of the optimization of the magnesium chloride concentrations for sequenced exon I of K-*ras* gene.

The magnesium chloride ($MgCl_2$) concentrations were adjusted to provide the best result. The $MgCl_2$ concentrations were varied from 1.0 mM to 3.0 mM respectively. The PCR products were subjected to electrophoresis on 2%(w/v) agarose gel in 0.5X buffer. Specifically sized of PCR product was obtained for the 260-bp.

Lane M = Standard DNA 100 bp molecular weight maker

Lane 1 = 1.0 mM of $MgCl_2$ concentration

Lane 2 = 1.5 mM of $MgCl_2$ concentration

Lane 3 = 2.0 mM of $MgCl_2$ concentration

Lane 4 = 2.5 mM of $MgCl_2$ concentration

Lane 5 = 3.0 mM of $MgCl_2$ concentration

3.3.2 The identification of mutation at codon 12 and codon 13 on exon I of the K-*ras* gene by using DNA sequencing technique

The amplified PCR products, which covering codon 12 and codon 13 on exon I of K-*ras* gene, were purification and performed DNA sequencing by dideoxynucleotides chain termination method. The nucleotides change at codon 12 and codon 13 on exon I of K-*ras* gene were compared with the normal sequences which derived form Gene Bank ID: L00045.

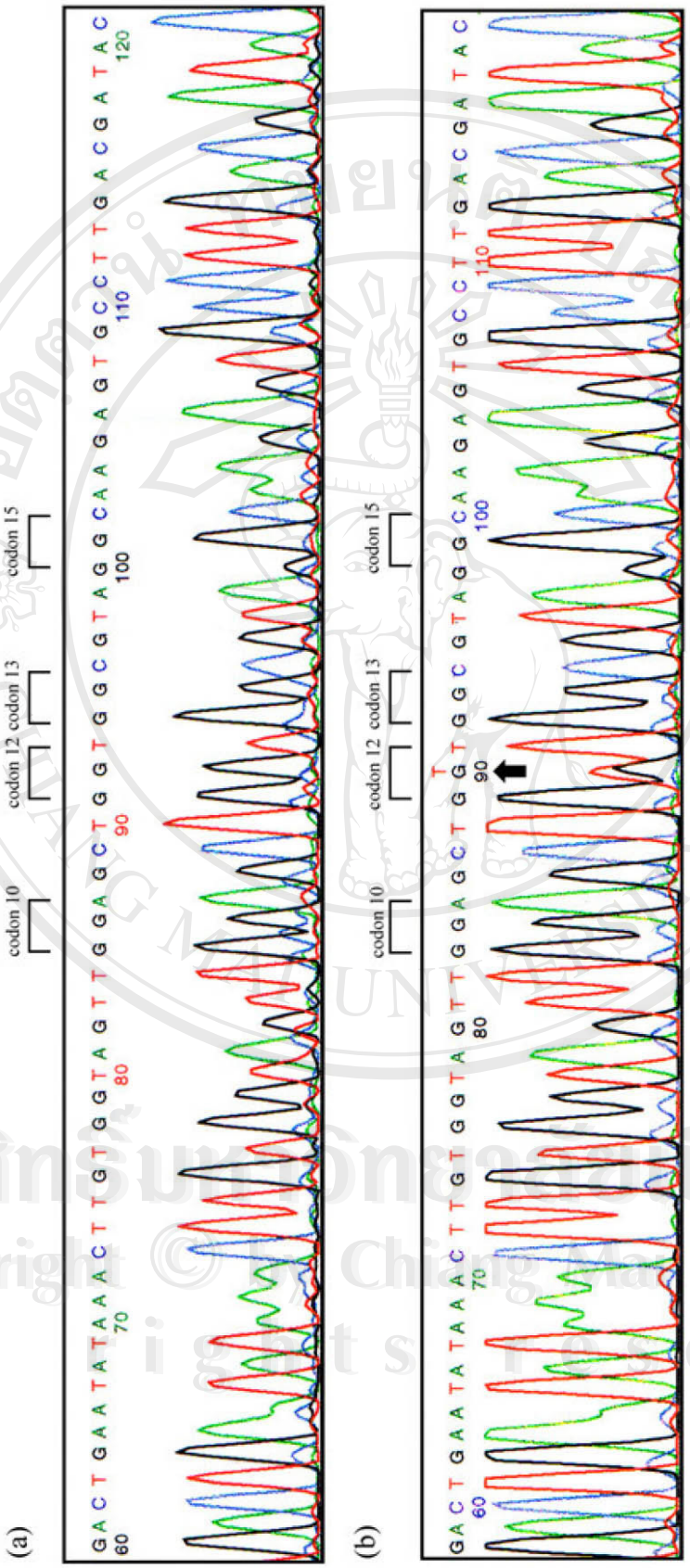

Figure 46 DNA sequence of exon I of K-*ras* gene from Thai ovarian cancer case OT023. **(a)** Normal sequence of exon I of K-*ras* gene. **(b)** The point mutation of GGT to GTT was detected in codon 12. Arrow indicates the point mutation.

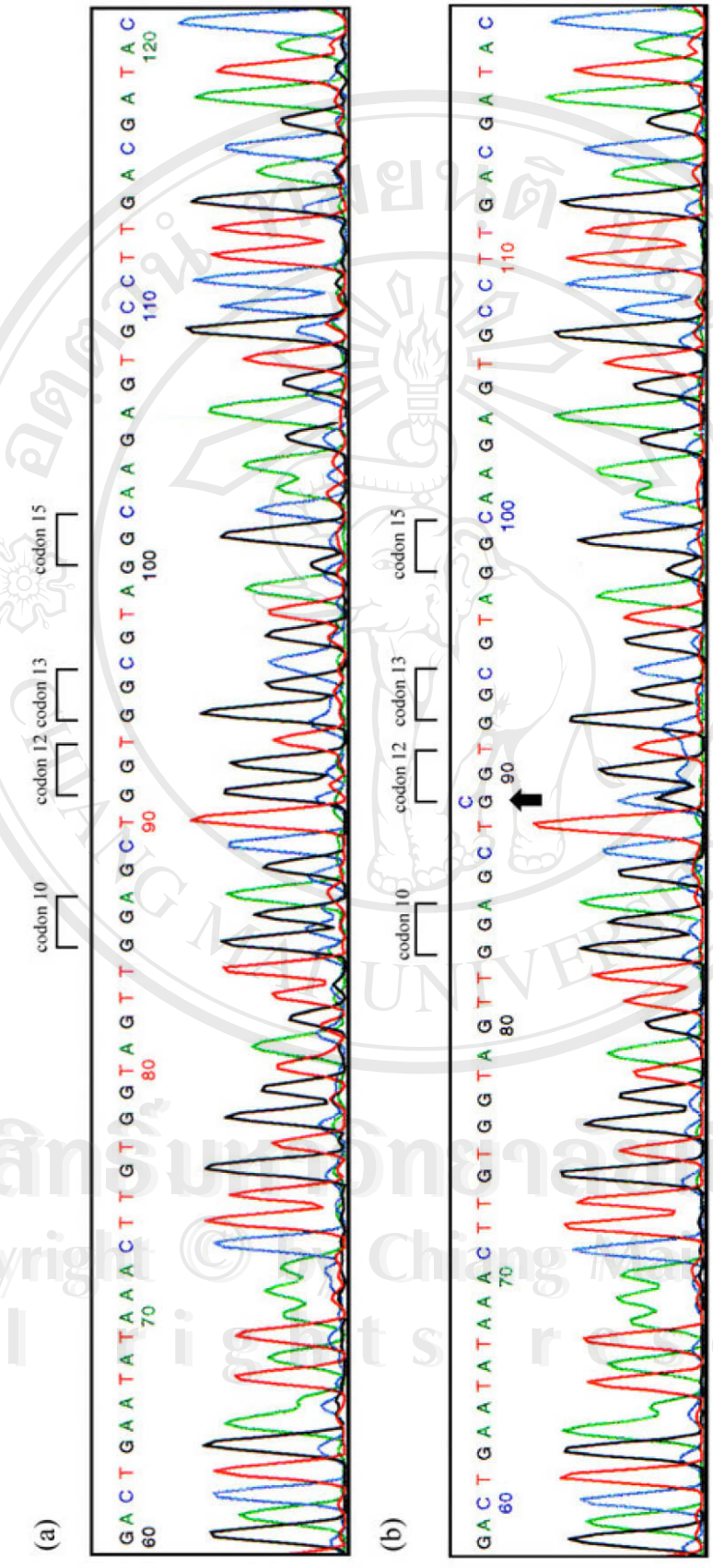

Figure 47 DNA sequence of exon I of K-*ras* gene from Thai ovarian cancer case OT057. **(a)** Normal sequence of exon I of K-*ras* gene. **(b)** The point mutation of GGT to CGT was detected in codon 12. Arrow indicates the point mutation.

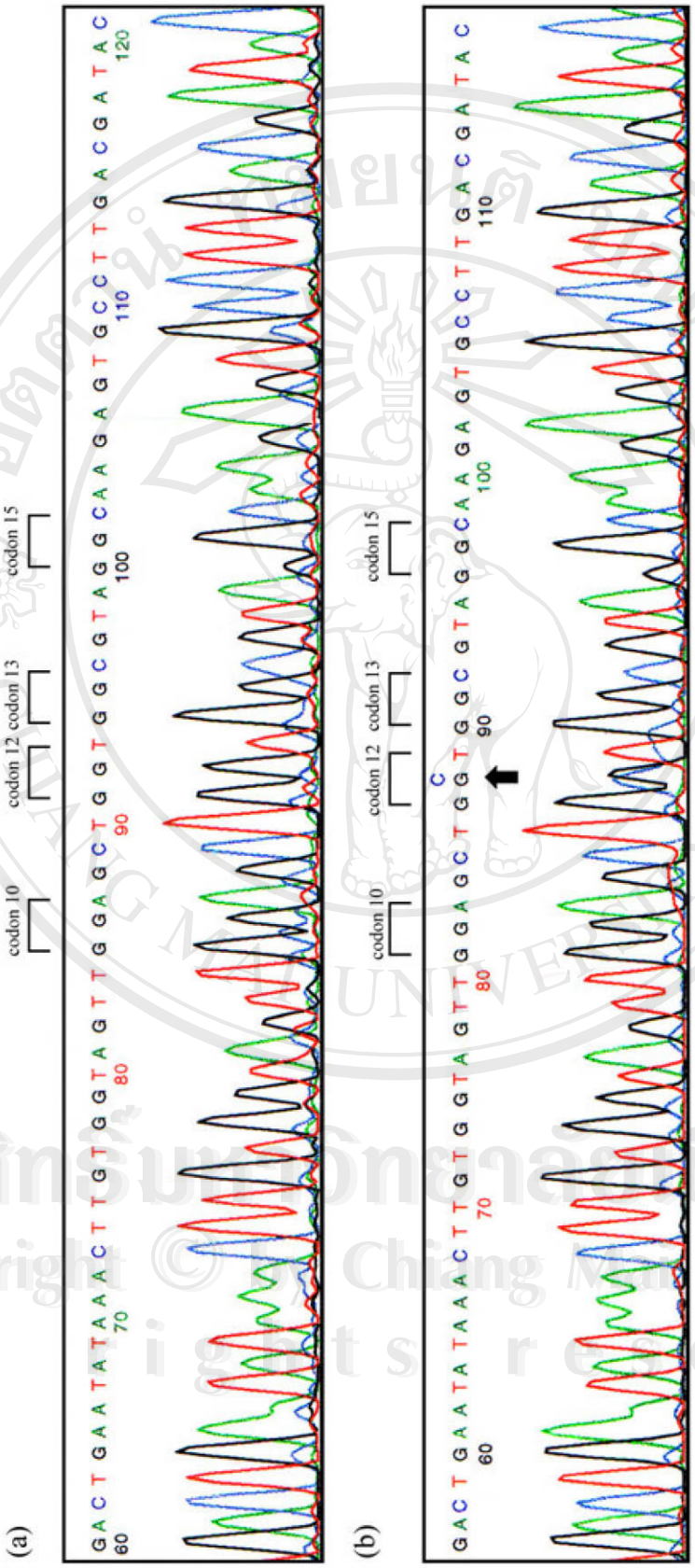

Figure 48 DNA sequence of exon I of K-*ras* gene from Thai ovarian cancer case OT060. **(a)** Normal sequence of exon I of K-*ras* gene. **(b)** The point mutation of GGT to GCT was detected in codon 12. Arrow indicates the point mutation.

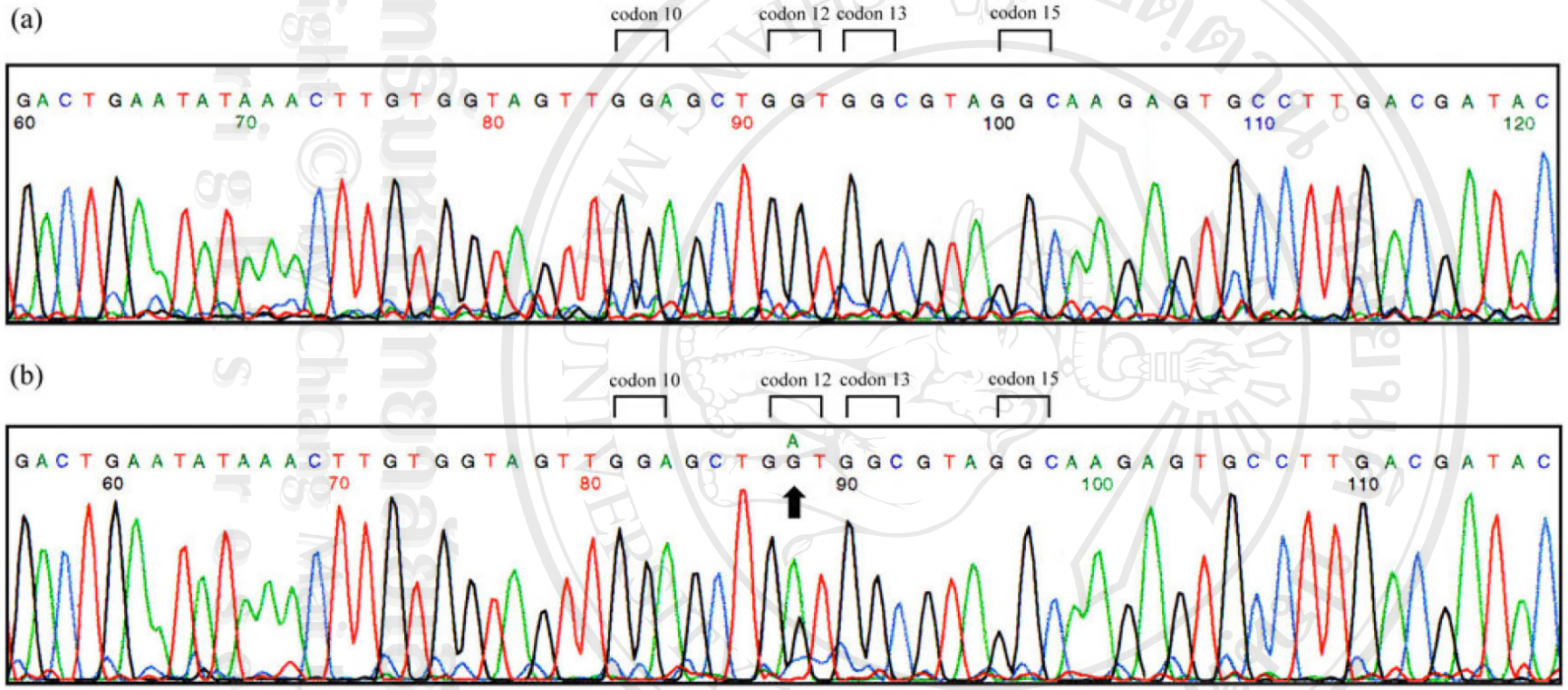

Figure 49 DNA sequence of exon I of K-*ras* gene from Thai ovarian cancer case OT069. **(a)** Normal sequence of exon I of K-*ras* gene. **(b)** The point mutation of GGT to GAT was detected in codon 12. Arrow indicates the point mutation.

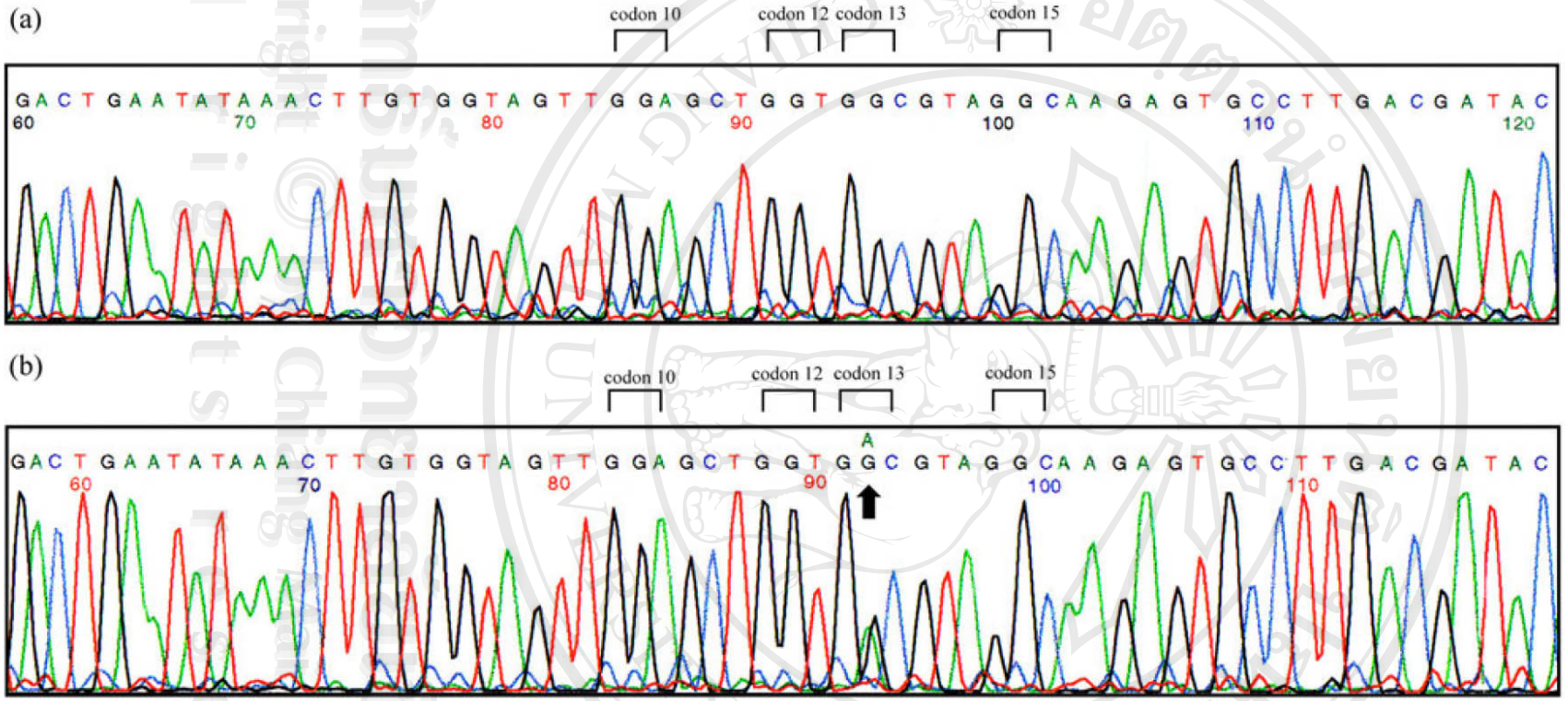

Figure 50 DNA sequence of exon I of K-*ras* gene from Thai ovarian cancer case OT026. **(a)** Normal sequence of exon I of K-*ras* gene. **(b)** The point mutation of GGC to GAC was detected in codon 13. Arrow indicates the point mutation.

Table 4 The clinicopathologic profiles and the results from the analyzation of mutations at codon 12 and codon 13 of K-*ras* gene by DNA sequencing in Thai ovarian cancer specimens.

DNA extracted form tissue	Age (year)	Histologic subtype	Tumor classification		K-*ras* mutation	
			LMP	Malignant	codon 12[a]	codon 13[b]
OT 003	49	Mucinous tumor of LMP	Low		GTT	
OT 011	50	Mucinous tumor of LMP	Low		GAT	
OT 023	37	Serous adenocarcinoma		1	GTT	
OT 026	51	Mixed epithelial of LMP	High			GAC
OT 036	73	Mucinous tumor of LMP	Low		GTT	
OT 038	17	Mucinous tumor of LMP	High		GTT	
OT 041	41	Mucinous adenocarcinoma		1	CGT	
OT 047	41	Mucinous tumor of LMP	Low		GCT	
OT 049	38	Endometrioid tumor of LMP*	Low		GAT	
OT 057	45	Mucinous tumor of LMP*	High		CGT	
OT 060	44	Matastatic adenocarcinoma			GCT	
OT 063	23	Serous tumor of LMP*			GAT	
OT 065	50	Mucinous tumor of LMP	High		GAT	
OT 069	30	Mucinous tumor of LMP	High		GAT	
OT 073	48	Mucinous adenocarcinoma		2	GCT	

[a] normal sequence of codon 12 GGT, [b] normal sequence of codon 13 GGC, and * tumor with microinvasion.

In the fifteen of K-*ras* mutations detected, 14 of 15 cases (93%) had the mutations at codon 12, including five (33%) involved a nucleotide change from GGT to GAT (Glycine⟶ Aspartate), four (27%) involved a change from GGT to GTT (Glycine⟶Valine), three (20%) involved a change from GGT to GCT (Glycine⟶Alanine), and two (13%) involved a change from GGT to CGT (Glycine⟶ Arginine) **(Table 5)**. One of 15 cases (7%) had codon 13 mutations detected, involved a nucleotide change from GGC to GAC (Glycine⟶Aspartate).

Table 5 Tumor histologic subtype and base substitution type at codon 12 and codon 13 of K-*ras* gene in Thai ovarian cancer tissues.

Tumor Histologic subtype	Total Mutant (case)	Type of Mutations				
		Codon 12 (GGT)				Codon 13 (GGC)
		CGT	GAT	GCT	GTT	GAC
Mucinous tumor of LMP	8	1	3	1	3	-
Mucinous adenocarcinoma	2	1	-	1	-	-
Serous tumor of LMP	2	-	1	-	1	-
Endometrioid tumor of LMP	1	-	1	-	-	-
Matastatic adenocarcinoma	1	-	-	1	-	-
Mixed epithelial tumor	1	-	-	-	-	1
Total cases	15	2	5	3	4	1