CHAPTER IV

RESULTS

1. Extraction of recombinant plasmids containing HPV type 6, 11, 16, 18, 31, 33, and 35 DNA from the transformed *E. coli*

After extraction and purification, the recombinant plasmids containing HPV type 6, 11, 16, 18, 31, 33, and 35 DNA was re-tested again by PCR. As expected, DNA fragments of 186 – 192 bps were observed from all plasmids. It was confirmed that all extracted plasmids contained HPV DNA and can be used in later experiments. The results were shown in Figure 14.

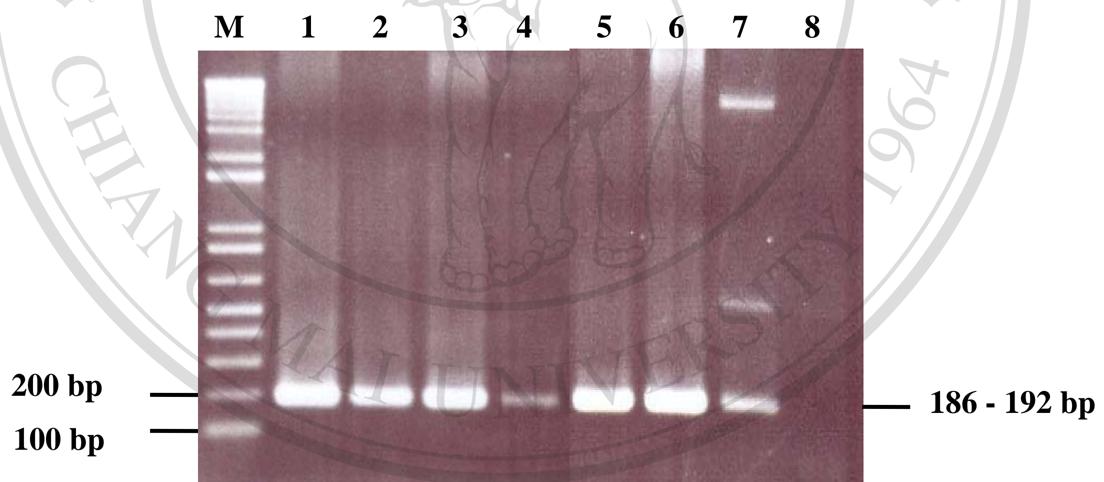

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Figure 14 The amplification of the plasmid HPV DNA fragments from colonies of transformed *E. coli* by PCR.

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Lane M; 1 Kb plus DNA marker Lane 1; plasmid DNA of HPV16,
Lane 2; plasmid DNA of HPV31 Lane 3; plasmid DNA of HPV33,
Lane 4; plasmid DNA of HPV35 Lane 5; plasmid DNA of HPV6,
Lane 6; plasmid DNA of HPV11 Lane 7; plasmid DNA of HPV18,
Lane 8; negative control using distilled water

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The plasmid DNA was then quantified by using the spectrophotometry. The quality of DNA was determined by the ratio between the O.D. at 260 and 280. The O.D. ratio of 1.8 or more indicates the high purity of DNA preparation. The results showed that all preparations had high yield and purity as the OD ratios were higher than 1.8. The performance of DNA from each preparation was shown in Table 6.

Table 6 Demonstrate the results of quantity and quality of HPV recombinant plasmids preparations.

Plasmid DNA	O.D.260	O.D.280	O.D.260/ O.D.280 ratio	Dilution factor	DNA μg/ml
HPV6	0.255	0.142	1.80	10	126
HPV11	0.209	0.109	1.92	10	105
HPV16	0.170	0.082	2.07	10	85
HPV18	0.237	0.120	1.98	10	119
HPV31	0.164	0.078	2.10	10	82
HPV33	0.301	0.144	2.09	10	151
HPV35	0.695	0.338	2.06	10	348

2. Determination the optimal conditions of TaqMan-based real-time PCR

2.1 Determination the optimal temperature of primers and probe

The concentration of primers and probe were optimized using recombinant plasmid HPV type 6, 11, 16, 18, 31, 33, and 35 as a target DNA. According to TaqMan-based real-time PCR technique, the optimal temperature for primers and probe annealing to target DNA was the most critical factor for successfully amplification and detection of the assay. This study, we determined the optimal annealing temperature between 50-60°C by using gradient temperature software. The software calculates and controls the decreasing or increasing of temperature in the gradient manner according to the number of columns or rows in the PCR block. Thus

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each column or row has temperature differ from another column or row nearby. When PCR reactions were performed with all 7 HPV recombinant plasmids at gradient temperature between 50-60°C, the optimal temperature was determined according to the C_t at which the amplify product was first detected. The results shown in Table 7 demonstrated that at temperature 53.7°C the DNA amplification was detected earlier (less C_t number) compared to at other temperature. This can be concluded that the optimal temperature for primers and probe was at 54°C and was used in next experiments.

Table 7 The C_t value from real-time PCR performing with gradient temperature between 50-60°C.

Temperature (°C) / HPV DNA	C_t value					
	52.0	**53.1**	**53.7**	**54.4**	**55.0**	**56.0**
HPV6	25.4	26.2	27.1	27.8	28.7	31.4
HPV11	20.4	21.0	21.6	22.7	23.3	25.3
HPV16	13.9	14.4	14.8	16.5	19.3	35.3
HPV18	12.7	13.0	13.1	12.9	13.3	14.4
HPV31	38.2	37.4	38.2	37.6	38.0	38.1
HPV33	27.6	28.5	29.2	30.3	32.0	33.5
HPV35	26.6	27.1	27.5	28.1	28.9	31.5

2.2 Determination of the optimal concentration of primers and probe

Since the forward primer, PGMY09/11, was the cocktail primers comprising 5 HPV specific primers; A, B, C, D, and E. To optimize the cocktail primers, each primer at concentration 80, 160 and 320 nM was mixed in an equimolar concentration. The PCR assay was performed with constant concentration (400 nM) of GP6+ reverse primer, 200 nM probe and 1 ng/µl of target HPV DNA. The results were shown in Table 8. When considered the C_t value obtained from those 3 concentrations of forward primers with all HPV types, they were not different. It meant that even at 80 nM of each primer in the cocktail was still sufficient for the

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reaction and not necessary to use the higher concentration. However, when considered the fluorescent signal curve, the curve from 80 nM primer was reached the plateau (exhausted) earlier than the others. So we decided that the concentration of primer at 160 nM each was the optimal and used for later experiment.

To optimized the concentration of TaqMan probe, the probe concentration was varied at 100, 200 and 400 nM and performed PCR assay with forward and reverse primers at concentration 160 nM each and 400 nM respectively. The results were shown in Table 9. At probe concentration 100 nM, the mean C_t value (19.64) was higher than those obtained from reaction with probe concentration 200 (17.38) and 400 nM (16.54). It meant that at probe concentration 100 nM was not optimal for the reaction. Moreover, probe concentration at 200 and 400 nM seemed to be saturated since the mean C_t value obtained were not much different. Taken together with cost of the assay, we considered that the optimal probe concentration should be at 200 nM.

Table 8 Results of C_t value obtained from PCR assay at different concentration of PGMY09/11 A-E (forward) primers with fixed concentration (400 nM) of GP6+ reverse primer.

HPV DNA \ PGMY09/11 concentration	C_t value		
	80 nM	**160 nM**	**320 nM**
HPV6	26.2	25.7	26.5
HPV11	21.1	21.0	21.4
HPV16	15.6	16.9	15.8
HPV18	12.4	12.2	13.3
HPV31	38.9	38.3	37.7
HPV33	30.8	29.6	28.4
HPV35	28.5	28.3	41.1

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Table 9 The results in C_t value obtained from each probe concentrations.

Probe concentration / HPV DNA	C_t value		
	100 nM	**200 nM**	**400 nM**
HPV16 (1st expt.)	19.91	16.93	16.19
HPV16 (2nd expt.)	19.37	17.83	16.8$_{8}$8
Mean C_t value	19.64	17.38	16.54

3. Determination of the sensitivity of TaqMan-based real-time PCR in detection of HPV DNA

The 10-fold serially dilutions of recombinant plasmid containing HPV16 DNA from concentration 100 ng/µl to 1 ag/µl were used to determine the sensitivity of the assays. Five microliters of each dilution was amplified and detected by TaqMan-based real-time PCR using the optimized conditions described above. Each set of experiment was performed in duplicates. The results were shown in Table 10. Since the baseline of real-time PCR assay is generally set at 40th C_t, any assays detected target DNA at C_t less than 40 were considered as positive results, in contrast, the assays that detected target DNA at C_t equal to or more than 40 were considered as negative results. The real time PCR assay optimized in this study could detect the lowest concentration of HPV DNA at 1 fg/µl with mean C_t value 39.5. While, at DNA concentration 100 ag/µl was detected at mean C_t value 42.5 which was below the baseline of the assay thus, considered as negative. According to this experiment, we concluded the sensitivity or lower detection limit of TaqMan-based real-time PCR was at 1 fg/µl. We further determined the precision of the test by performing the inter-run assay with 1 fg/µl HPV16 DNA for 10 times. The results showed that HPV DNA could be detected in all runs at C_t value 38.02-39.72 (mean C_t value 39.3). It was confirmed that HPV DNA at concentration 1 fg/µl or 5 fg/reaction was the lower detection limit of this assay.

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Table 10 The results in C_t value of the sensitivity determination of TaqMan-based real-time PCR in detection of plasmid HPV16 DNA.

HPV DNA concentration	Mean C_t Value	
	1st experiment	2nd experiment
1 ng/μl	17.0	16.3
100 pg/μl	21.3	20.6
10 pg/μl	24.4	23.6
1 pg/μl	28.5	28.6
100 fg/μl	32.2	33.2
10 fg/μl	34.8	34.4
1 fg/μl	**39.7**	**39.3**
100 ag/μl	42.1	42.9

4. Detection of HPV DNA in cervical specimens by using TaqMan-based real-time PCR

4.1 Determination of the optimal concentration of Proteinase K and incubation time for DNA extraction method

Since the cervical samples were preserved in *Liqui*-PREP™ solution, the DNA extraction method was carefully considered in this study. Concerning with the easy, simplicity and cost saving of the assay, we decided to use lysis buffer with proteinase K to lyses cervical cells and collected the crude DNA lysate for PCR assay. The concentration of proteinase K were optimized with lyses time and the β–globin DNA was used as target for PCR. After PCR, the amplify products were detected by agarose gel electrophoresis. The lowest concentration of proteinase K with the shortest lyses time using for crude DNA extraction that allowed detection of β–globin DNA clearly. The results were shown in Figure 15 (A-D). The amplify DNA band

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on the agarose gel was observed in all concentration of proteinase K and all incubation time, although with varying intensity. It was confirmed that the crude DNA lysate obtained from this method was qualified for PCR assay. However, concerning with the quantity of DNA in the lysate, the intensity of amplify DNA band was carefully considered. It was shown that the proteinase K at concentration 100 μg/ml (figure 16 A) give the faith band of DNA at every incubation time compared to other concentrations; 200, 300 and 400 μg/ml (Figure 15 B, C, and D). Although, proteinase K at concentration 400 μg/ml gave the strongest intensity of DNA band even with short incubation time as 15 min. However, at concentration 200 μg/ml, the clearly DNA band was observed only at the incubation time 120 and 180 minutes. With proteinase K concentration 300 μg/ml, the high intensity of DNA band was observed in all incubation time but lower intensity than at 400 μg/ml. Consideration based on cost saving and less time consuming, we decided to use proteinase K concentration 200 μg/ml and 30 minutes incubation time as a DNA extraction protocol in this study.

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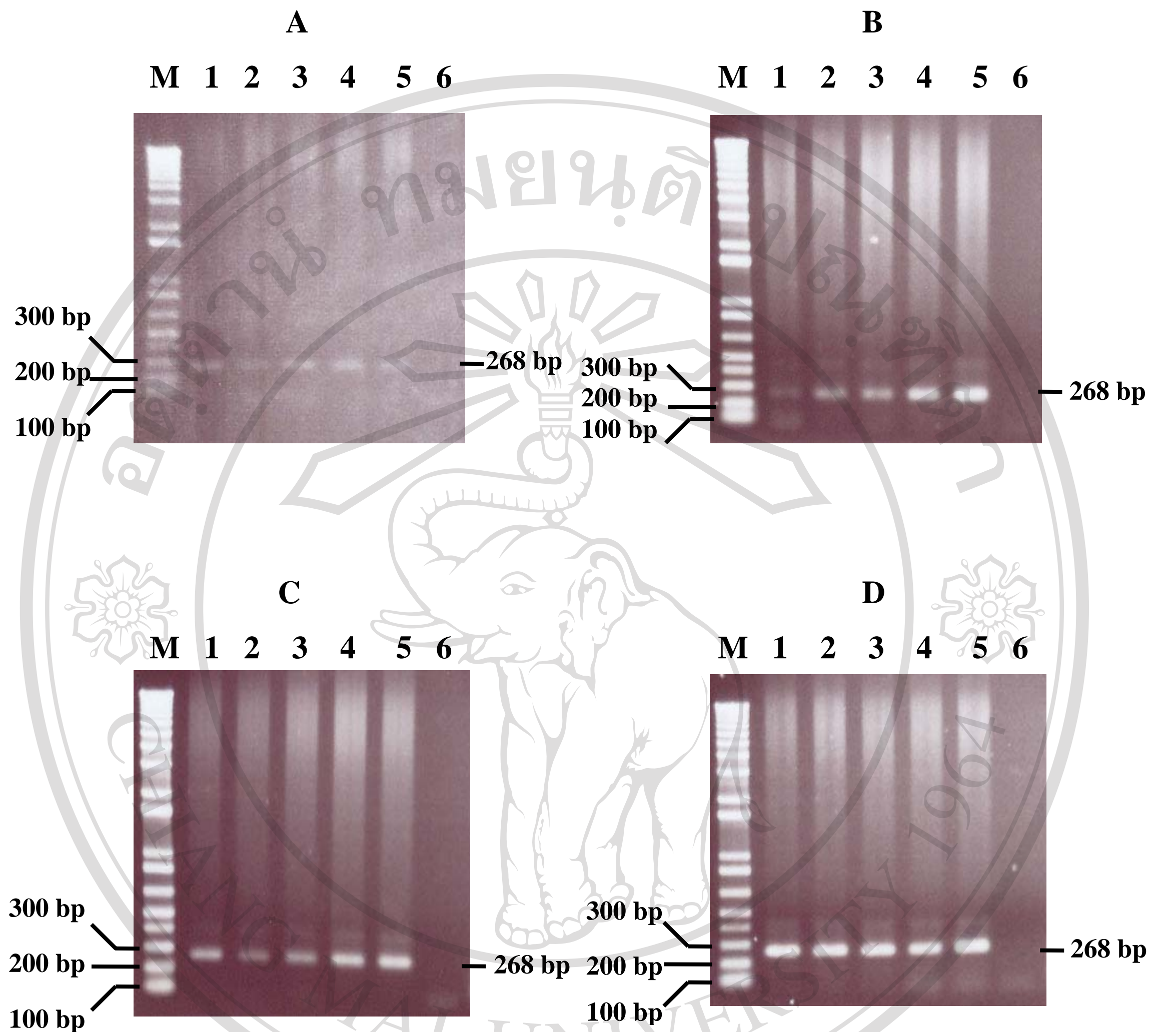

Figure 15 Illustrations the agarose gel electrophoresis of PCR amplification products of β–globin gene extracted from cervical samples using different concentrations of proteinase K and lyses time.

A; proteinase K 100 μg/ml B; proteinase K 200 μg/ml

C; proteinase K 300 μg/ml D; proteinase K 400 μg/ml

Lane M; 1 Kb plus DNA marker

Lane 1 – 5; lyses time at 15, 30, 60, 120, and 180 minutes respectively

Lane 6; negative control using distilled water

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4.2 Detection of HPV DNA by using TaqMan-based real-time PCR

Four-hundred and fifty three cervical scrapes randomly collected from women with VIA positive (213 samples) and VIA negative VIA (240 samples) were tested for HPV DNA by using TaqMan-based real-time PCR optimized in this study. All cervical cell scrapes were extracted for crude DNA lysates by using protocol optimized in 4.1 (lysis buffer containing Proteinase K at 200 µg/ml and incubation time 30 minutes). After detection, the HPV positive samples were genotyping by REA technique and all negative samples were confirmed by detecting the β–globin DNA.

Among 453 samples, HPV DNA was detected in 52 and 401 negative samples were confirmed with β–globin DNA. There were 89 samples lacking β–globin DNA after testing and were excluded from later analysis. It was possible that those DNA extracts containing some PCR inhibitors or loss of DNA during extraction. After exclusion, there were totally 364 samples; 191 VIA positive and 173 VIA negative, were used for analysis (Table 11). From 364 cervical samples, 52 (14.29%) were positive for HPV DNA and 312 (85.71%) were negative. When considered together with VIA testing, it is interesting that the number of HPV DNA positive in VIA positive women was higher than those in VIA negative; 18.85% vs. 9.25%.

Table 11 Comparison between TaqMan-based real-time PCR and visual inspection with acetic acid (VIA) in detection of HPV DNA from cervical cell scrapes.

Visual inspection with acetic acid (VIA)	**Number of samples (n)**	**HPV DNA**	
		Positive	**Negative**
Positive	191	36 (18.85%)	155 (81.15%)
Negative	173	16 (9.25%)	157 (90.75%)
Total number	364	52 (14.29%)	312 (85.71%)

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5. Detection of HPV genotypes by restriction enzyme analysis (REA)

All HPV DNA positive samples were analyzed further for HPV genotypes using the REA technique. After PCR products were digested with 3 restriction enzymes; *Mae*III, *Rsa*I and *Mse*I, the digested products were visualized by agarose gel electrophoresis. For genotype analysis, all REA fragments size was compared with the REA fragments size in table 6 which obtained from the known standard genotypes.

Among 52 HPV positive samples, 12 different types of HPVs were observed, they were HPV6, 16, 18, 31, 35, 39, 51, 52, 58, 59, 66, and 72. Among these, all except 2 genotypes; HPV6 and 72 are HPV low risk types. The results were summarized in Table 12 and Figure 16. Unfortunately, there were 9 samples that gave unclear REA patterns, which prevented proper identification. It is possible that a mixed infection might have occurred in those cases. However, among the clearly identified HPV types, HPV16 was the most frequently found (22 of 43 samples; 51.16%) followed by HPV18 (18.60%). HPV51, 58, and 59 were each found in 2 (4.65%) samples. In VIA positive samples, HPV16 was the most prevalent which represented 55.17% of all HPV types identified followed by HPV 18 (17.24%). The HPV low risk type, HPV 6 was detected in 1 sample. Among VIA negative samples, HPV16 was also found in most prevalent (42.86%). However, HPV72, the HPV low risk types was detected in 1 sample.

Table 12 Genotype distribution of HPV as determined by PCR-REA.

VIA	**Number of samples (n)**	**HPV types**												
		6	16	18	31	35	39	51	52	58	59	66	72	NT
Positive	36	1	16	5	1	1	1	-	-	2	2	-	-	7
Negative	16	-	6	3	-	-	-	2	1	-	-	1	1	2
Total number	52	1	22	8	1	1	1	2	1	2	2	1	1	9

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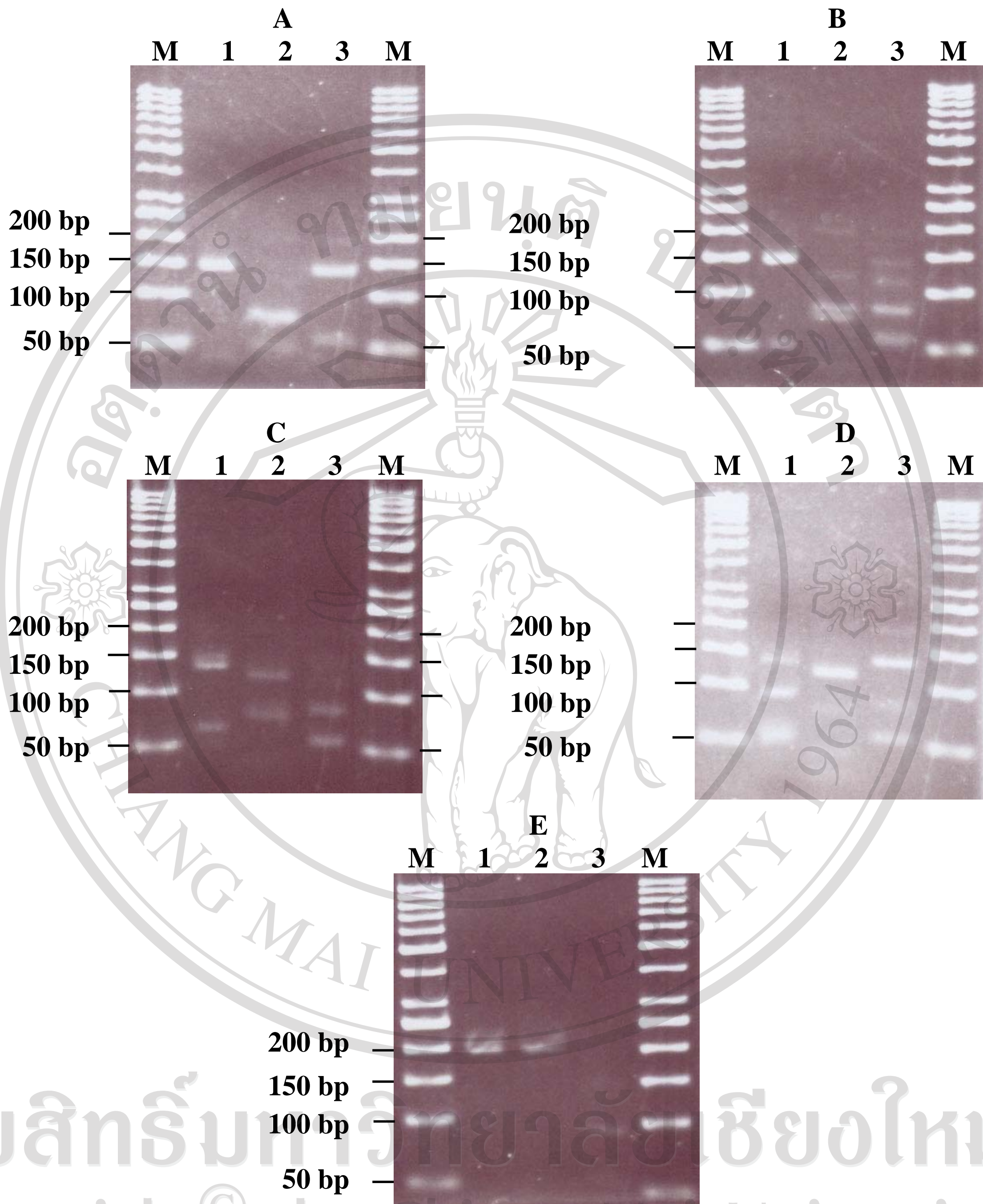

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Figure 16 The illustration of PCR-REA patterns of HPV genotypes from cervical cell scrapes.

A; HPV 16 (sample No. 144) B; HPV18 (sample No. 28)

C; HPV51 (sample No. 20) D; HPV58 (sample No. 55)

E; HPV59 (sample No. 213) Lane M; 50 bp DNA marker.

Lane 1 – 3; PCR products digested with *Mae*III, *Rsa*I and *Mse*I respectively