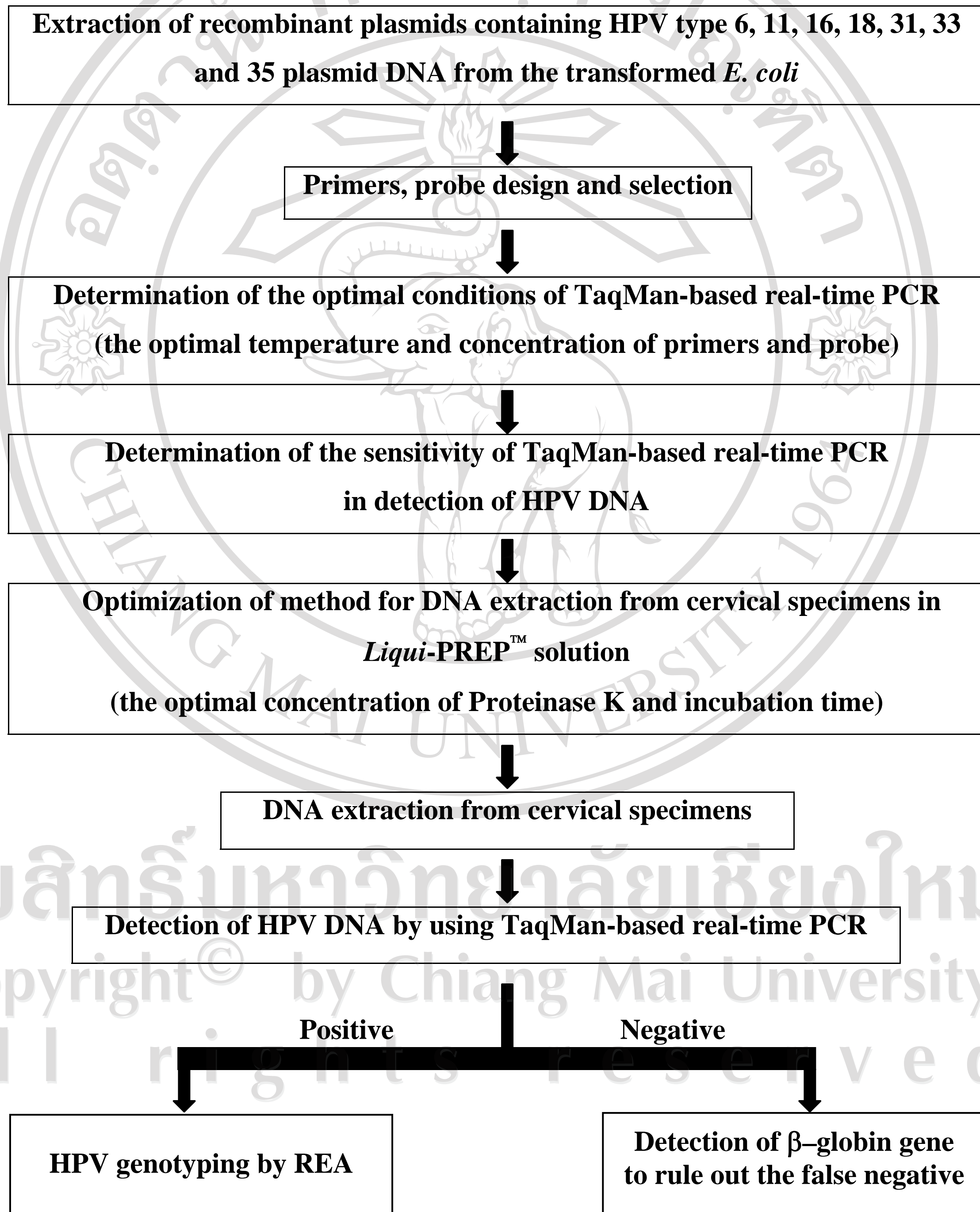
CHAPTER III

RESEARCH DESIGN, MATERIALS AND METHODS

1. Research design



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2. Materials and methods

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

Recombinant plasmids containing HPV DNA type 6, 11, 16, 18, 31, 33, and 35 in transformed *E. coli* were kindly provided by Dr. Pranee Leechanachai at Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. These plasmids were used for optimization and determination the sensitivity of the assay and also as a positive control DNA. The plasmid DNA was extracted from transformed *E. coli* by minipreparation using QIAprep Spin Miniprep Kit (Qiagen, Germany).

The procedure of purification was performed as recommended in the manufacturer's instruction manual. Briefly, a single bacterial colony was picked up and cultured in 5 ml of LB medium containing 100 μg/ml of ampicillin in a loosely capped 15 ml tube and incubated at 37°C for 12-16 hours with vigorous shaking. After incubation, the culture was transferred into a sterile 1.5 ml microtube and centrifuge at 6,800 x g for 3 minutes at room temperature (15-25°C). The supernatant was removed and the bacterial pellet suspended in 250 μl of Buffer P1 (resuspension buffer). Then, 250 μl of Buffer P2 (lysis buffer) was added and mixed the contents by inverting the tube (saw solution turned blue). After mixing, 350 μl of Buffer P3 (neutralization buffer) was added and mixed the contents immediately by inverting the tube (solution now turned colorless). After centrifuged at 12,000 x g for 10 minutes, the supernatant was collected and applied to the QIAprep spin column set and centrifuged at 12,000 x g for 60 seconds. The plasmid DNA was bound to the glass fiber in the QIAprep spin column. The bound plasmid DNA was washed twice with Buffer PB and Buffer PE to remove the protein and other cellular contents and the plasmid DNA was eluted with 50 μl of Buffer EB and stored at -20°C until further examination.

The concentration of the plasmid DNA was determined by using the spectrophotometry method. The DNA was diluted in distilled water at an appropriate dilution and the optical density (O.D.) was then measured for nucleic acid and protein

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at a wavelength of 260 and 280 nm, respectively, using a UV spectrophotometer. The quantity of DNA was calculated by using the following equation:

Quantity of DNA (ng/μl) = $O.D._{260}$ x dilution factor x 1 $O.D._{260}$ unit of double-stranded DNA concentration.

= O.D. at 260 nm x dilution factor x 50

1 $O.D._{260}$ unit contains double-stranded DNA = 50 ng/μl

The purity of plasmid DNA preparation could be estimated by the ratio between O.D. 260 and 280. Pure preparation of DNA has the ratio O.D. 260/O.D. 280 values of 1.8 or more. If the ratio value had been lower, the preparation would have contained some contaminants (*e.g.*, protein). The DNA concentration (ng/μl) can be converted into a number of copies by using the mathematical formulas as follows:

$$\frac{6 \times 10^{23} \text{ (copies/mol)} \times \text{concentration (g/}\mu\text{l)}}{\text{MW (g/mol)}} = \text{amount (copies/}\mu\text{l)}$$

MW = (number of base pair) x (660 daltons/base pairs)

1 mol = 6×10^{23} molecules (copies)

2.2 Designation, selection, and determination of the optimal conditions of TaqMan-based real-time PCR

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2.2.1 Primers, probe designation and selection

The PGMY11-A, B, C, D and E primers, which had been published earlier were selected as a forward primers (89). While the GP6+ and GP5+ primers published by Jacobs *et al*. (88) were modified and used as reverse primer and probe respectively. Those primers franking 186-192 bp region and probe were highly specific to the L1 ORF of almost all mucosal HPV types. All primers and probe chosen above were modified and retested for specificity by aligning with complete L1 genome sequences of HPV type 1-107 downloaded from GenBank

(http://www.ncbi.nlm.nih.gov) using alignment programs; Bioedit, ClustalX and Fast PCR Software. Primers and probe were also tested according to the TaqMan system before ordering for synthesis.

The modified GP5+ probe (TaqMan probe) was labeled with the fluorescent dye; FAM (6-carboxyfluorescein) as reporter dye at 5' end and BHQ1 (black hole quencher 1; amino-modifier C6 dT) as quencher dye at 3' end. Synthesis was performed by Operon Biotechnologies (Cologne, Germany). Aliquots of the primers and probes were stored at -20 ºC. The location of primers and probe were shown in Figure 13.

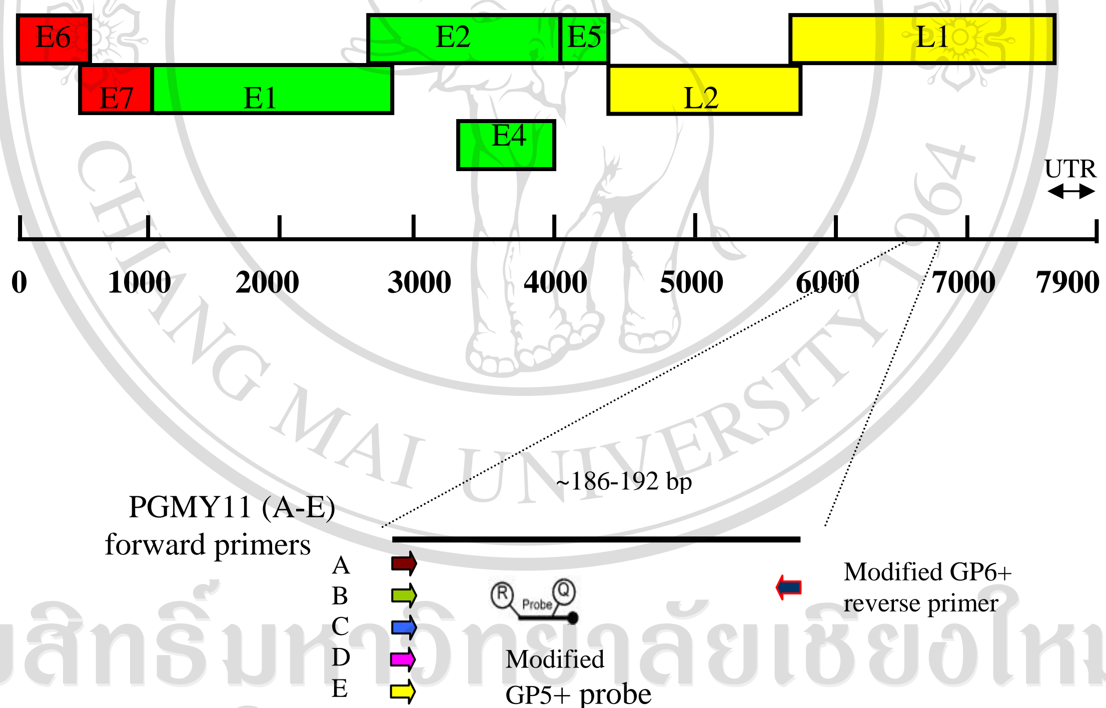

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Figure 13 The location of PGMY11-A to E forward primers, modified GP6+ reverse primer and modified GP5+ HPV Probe, contains a reporter (R) and quencher (Q) dye on L1 gene.

2.2.2 Determination of the optimal conditions of TaqMan-based real-time PCR

The factors involving PCR reaction including primers-probe concentrations, annealing temperature were optimized according to TaqMan-based real-time PCR. FastStart Universal Probe Master Mix (Roche Applied Science, Penzberg, Germany) was used for PCR reaction performing as recommended by manufacture.

2.2.2.1 Determination of the optimal annealing temperature of primers and probe

The optimal annealing temperature of primers and probe were determined by performing PCR at a gradient of annealing temperature. The temperature at which PCR product was highest produced was determined as an optimal temperature. The experiment was performed as described below. The extracted HPV6, 11, 16, 18, 31, 33, and 35 containing plasmid DNAs from 2.1 were diluted into 1 ng/µl in steriled distilled water. PCR mixture consisted of 12.5 µl of 2X FastStart Universal Probe Master containing PCR buffer, dATP, dGTP, dCTP, dUTP, and FastStart *Taq* DNA polymerase, primers and probe concentrations were used as recommended by manufacture, in a total volume of 20 µl/reaction. Then, 5 µl of extracted plasmid DNA (1 ng/µl) was added. The amplification was performed in Chromo4™ Real-time PCR instrument (Bio-Rad Laboratories, USA) with the following PCR cycle; 10 minutes at 95°C for FastStart *Taq* DNA polymerase activation and template denaturation, followed by 44 cycles of 15 seconds at 94°C, and the gradient of annealing temperature from 50-60°C for 90 seconds. PCR product was analyzed as an increase in fluorescence using Opticon Monitor 3 software.

Analysis based on real time PCR assay, the fluorescent signal intensity is increasing in direct proportion to the amplify product and the positive amplification signal is determined according to the amplification cycle (cycle threshold; C_t) at which the fluorescent signal coming up above the baseline. Thus, the amount of target DNA or amplify product is inversely proportion to the number of C_t.

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2.2.2.2 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 was mixed in an equimolar concentration indicated in Table 3. The PCR assay was performed with constant concentration (400 nM) of GP6+ reverse primer, 200 nM probe and 1 ng/µl of extracted plasmid DNA containing HPV6, 11, 16, 18, 31, 33, and 35 from 2.1. PCR mixture was performed as described in 2.2.2.1 The amplification was done with annealing temperature that had been optimized in 2.2.2.1. All targets DNA concentration was run in duplicate.

The lowest concentration of forward primers that allowed the amplify products be detected earliest (low C_t number) is determined as the optimal concentration and used for optimized the concentration of TaqMan probe. The probe concentration was varied at 100, 200, and 400 nM and performed PCR assay with constant concentration (400 nM) of reverse primer and 1 ng/µl of extracted plasmid HPV16 DNA. PCR mixture and detection were performed as described above. The test was run in duplicate.

The lowest concentration of probe that allowed low C_t number is determined as the optimal concentration and used in the later experiments.

Table 3 The ratio of concentrations between forward primers and reverse primer used in TaqMan-based real-time PCR

Forward/ reverse primers (nM)	**Forward/ reverse primers (nM)**	**Forward/ reverse primers (nM)**
(80, 400)	(160, 400)	(320, 400)

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2.3 Determination of the sensitivity of TaqMan-based real-time PCR for detection of HPV DNA

To determine the sensitivity or lower detection limit of the assay, extracted plasmid with HPV16 DNA was 10-fold serially diluted in steriled distilled water from 100 ng/µl to 1 ag/µl and used in the experiment. PCR reaction was performed with the optimized conditions; annealing temperature and primer-probe concentrations, as described in 2.2.2.1 and 2.2.2.2. The varying concentrations of target DNA; 100 ng/µl to 1 ag/µl were added to each tube. All dilutions of DNA were run in duplicate. The lowest concentration of target DNA that can be detected was determined as the lower detection limit of the assay.

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

2.4.1 Specimen collection and storage

A total of 453 liquid-based cytological specimens preserved in *Liqui*-PREP™ solution (LGM international, Inc., Fort Lauderdale, FL, USA) were collected from women attending clinics for routine cervical screening or during follow-up after treatment at the Sexually Transmitted Infection Center 10 Chiang Mai during June to August 2006. Specimens were firstly screened by Pap smear for abnormal cervical cells then the leftover samples were stored at 4°C for HPV DNA detection later. After cervical specimen collection for Pap smear, visual inspection with acetic acid (VIA) was examined on uterine cervix with 3-5% acetic acid by physician. A positive result is the present of acetowhite lesions around the cervix, while no changing of the tissue in negative result.

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2.4.2 DNA extraction from cervical cells preserved in *Liqui*-PREP™ solution

Liqui-PREP™ is a fixative solution for well-preserved cellular morphology for monolayer preparation of cervical cells. After fixation, they might have some

properties changed especially cell and nuclear membrane, method for extraction of DNA should be carefully considered. In this study, we aimed to use the DNA extraction method that was simple, less time consuming and cost saving, thus, we used lysis buffer with proteinase K to lyses cells. To obtain the best result, concentration of proteinase K and incubation time were optimized before application.

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

Ten milliliters of pooled cervical cells preserved in *Liqui*-PREP™ was centrifuged at 2,900 x g for 15 minutes at room temperature. After the supernatant was discarded, the pellet were washed twice with 4 ml of steriled PBS and centrifuged at 1,500 x g for 10 minutes at room temperature. Then, transferred cell pellet into a sterile 1.5 ml and re-centrifuged at 10,000 x g for 5 minutes. After supernatant was discarded, 100 µl of lysis buffer containing varying concentration of proteinase K at 100, 200, 300, and 400 µg/ml was added. Each concentration was performed in 10 replicated tubes. After mixing by vortexing vigorously, they were incubated at 60°C in water bath for 15, 30, 60, 120, and 180 minutes (Table 4). The proteinase K was later inactivated at 96°C for 15 minutes in dry heat block. The reaction tubes were finally centrifuged at 10,000 x g for 1 minute and transferred the supernatant into a new sterile 1.5 ml microcentrifuge tube and stored at -20°C. To determine the optimal extraction condition, the human β–globin DNA in the extractions was chosen as a target for analysis by PCR. The lowest concentration of proteinase K with the shortest incubation time that gave the maximum amplify product is deciding as the optimal condition for DNA extraction procedure and used for next experiment.

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Table 4 The varying concentration of proteinase K and incubation time used for extraction of DNA from preserved cervical cells.

Incubation time (min) \ Proteinase K (µg/ml)	100	200	300	400
15	Tube 1, 2	Tube 11, 12	Tube 21, 22	Tube 31, 32
30	Tube 3, 4	Tube 13, 14	Tube 23, 24	Tube 33, 34
60	Tube 5, 6	Tube 15, 16	Tube 25, 26	Tube 35, 36
120	Tube 7, 8	Tube 17, 18	Tube 27, 28	Tube 37, 38
180	Tube 9, 10	Tube 19, 20	Tube 29, 30	Tube 39, 40

2.4.3 DNA extraction from cervical specimens

Cervical cell scrapes in Liqui-PREP™ left-over from routine cytological examination were extracted for DNA using condition that had been optimized in 2.4.2.1. Before extraction, cervical cells were well dispersed by vortexing for 10-15 seconds and discarded the Rovers® Cervex-Brush™. Three hundred microliters of cell suspension was transfered to a sterile 1.5 ml microcentrifuge tube (2-6 tubes for each samples) and centrifuged at 8,000 x g for 1 minute and discarded the supernatant. Then, washed twice with 1 ml of steriled 1x PBS, cell pellets were kept at -20°C until used.

For DNA extraction, the frozen cell pellets were thawed at room temperature for 15 minutes and lysed by adding 100 µl of lysis buffer containing proteinase K and incubated as indicated in 2.4.2.1. The supernatant was aliquoted into 2 sterile tubes and kept at -20°C until tested for HPV DNA.

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

PCR reaction mixture was performed with the optimal condition as described in 2.2.2.1 and 2.2.2.2. Five microliters of DNA extracted in 2.4.3 was added into each reaction tube. Recombinant plasmid HPV11 DNA at the concentration of 1 pg/µl and distill water were used as positive and negative control respectively. To exclude false negative results, 5 µl of DNA extract from all negative samples were tested for presence of β–globin DNA by conventional PCR with primers specific to β–globin gene. Samples that positive with β–globin DNA amplification were confirmed negative results while β–globin DNA negative samples indicated as false negative results due to presence of PCR inhibitor or no DNA in the extracts.

2.5 Detection of HPV genotype by restriction enzyme analysis (REA)

As the highly polymorphism of HPV nucleotides was observed in L1 region, thus, it was selected as a target for genotyping by many authors and our study as well.

2.5.1 Restriction enzyme selection

NEB cutter program was used to select the restriction enzyme (http://tools.neb.com/NEBcutter) for genotyping of HPV. Different types of HPV DNA sequences were downloaded from GenBank (http://www.ncbi.nlm.nih.gov). The NEB cutter program was applied to each DNA sequence franking by primers used in PCR assay. The restriction enzymes which can differentiate most genotypes of HPV were selected and each restriction fragment sizes were calculated and tabulated as a standard fragment size table for genotyping experiments. The REA standard fragment size table of L1 region was shown in table 5.

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2.5.2 HPV genotyping by REA

2.5.2.1 Amplification of HPV DNA positive samples by using conventional PCR

Since the FastStart Universal Probe Master Mix used in real time PCR comprised uracil instead of thymidine that make the amplify DNA fragments can not be digested by the thymidine dependent restriction enzymes. Thus for genotyping, all samples positive for HPV DNA were re-amplified by conventional PCR before digested with appropriate enzymes. The PCR reaction mixture comprising PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, and 0.1% Triton X-100), 1.5 mM of $MgCl_2$, 200 μM of each deoxynucleotide triphosphates (dNTPs; dATP, dCTP, dGTP, and dTTP), 10 pmol of each PGMY11 A-E forward primer pools, 50 pmol of modified GP6+ reverse primer and 5 U of *Taq* DNA polymerase (New England Biolabs, USA). Five microliters of extracted DNA was added to the PCR mixture with final volume of 50 μl. The amplification was performed in an oil-free thermal cycler (MJ-Biorad, USA). The PCR cycle was as the following; denatured at 94°C for 1 minute, annealed at 50°C for 1 minute and extension at 72°C for 1 minute. The cycle program was repeated for 30 cycles and followed by another 7 minutes at 72°C to ensure the complete extension.

The amplified product was detected by electrophoresis on 1% agarose gel in 0.5x TBE buffer at 100 volts for 30 minutes. After electrophoresis, the agarose gel was stained with 2 μg/ml of ethidium bromide, destained in distilled water, visualized and photographed under UV-transilluminator. The amplified product was later purified and used for genotyping.

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2.5.2.2 Purification of the PCR product

The amplified product of the L1 HPV region from 2.5.2.1 was purified by using the High Pure PCR Product Purification Kit (Roche Applied Science, Germany). The procedure of purification was performed as the procedures recommended in the manufacturer's instruction manual. Briefly, the amplified DNA fragment separated on 1% agarose gel electrophoresis was excised by using a sterile

scalpel under ultraviolet light. The gel slice was incubated in 3 gel volumes (weight by volume) of Binding Buffer at 56°C for 10 minutes. After the gel slice was dissolved completely, 1.5 gel volume of isopropanol was added and then applied the mixture to High Pure filter tube. The filter tube was centrifuged at 10,000 x g for 1 minute and discarded the supernatant. The DNA was selectively bound to the special glass fibers pre-packed in the preforward of the chaotropic salt guanidine thiocyanate of the High Pure filter tube. Then, 500 µl of Wash Buffer was added into the filter tube and centrifuged for 1 minute and the supernatant was then removed, as before. The DNA was purified in a series of rapid wash-and-spin steps to remove contaminating primers, nucleotides and salts. After that, 200 µl of Wash Buffer was added to ensure optimal purity and complete removal of Wash Buffer from the glass fibers. Finally, 50 µl of Elution buffer was added to elute the DNA.

2.5.2.3 HPV Genotyping by REA

The purify products from 2.5.2.2 were then digested with 3 restriction enzymes selected in 2.5.1; *Mae*III, *Rsa*I and *Mse*I. The reaction mixture for all restriction enzymes was preformed as recommended by manufacture and 1-2 µg of L1 gene amplified product in a total volume of 25 µl for *Mae*III and 20 µl for *Rsa*I and *Mse*I. After mixing by brief centrifugation, the mixture was incubated in water bath at 55°C for 4 hours for *Mae*III and 37°C for 4 hours for *Rsa*I and *Mse*I.

The digested products were analyzed on 3% agarose gel electrophoresis in a 0.5x TBE buffer at 100 volts for 1 hour. After electrophoresis, the gel was stained with 2 µg/ml of ethidium bromide, destained in distilled water, visualized and photographed under UV transilluminator. The DNA fragment size was determined according to standard DNA size marker, 50 bp DNA Ladder GeneRuler™ (Fermentas, USA). To obtain the genotyping result, the patterns of digested DNA size fragments were compared with the standard table (table 5) constructed in 2.5.1. In the case of HPV 26, 31, 33, 35, 45, and 56 which gave a similar pattern, in order to differentiate further, the *Sfc*I restriction enzyme was used in addition to those 3 enzymes.

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Table 5 The L1 REA genotyping patterns constructed according to the reference HPV sequences obtained from Genbank.

HPV type	Digested fragment size (bp)			
	Mae III	*Rsa* I	*Mse* I	*Sfc* I
6	89, 51, 46	73, 67, 46	186	
11	140, 46	113, 73	186	
16	143, 32	73, 70, 46	136, 54	
18	146, 46	81, 73, 38	80, 58, 54	
26	146, 46	101, 73	123, 69	100
31	143, 46	116, 73	120, 43	185
33	82, 51, 46	74, 73	132, 54	167
35	82, 61, 32	74, 73, 42	135, 43	138, 51
39	192	119, 73	138, 54	
45	146, 46	74, 73, 45	80, 58, 54	141, 51
51	120, 61	116, 73	80, 55, 54	
52	140, 46	186	100, 54	
53	140, 46	186	132, 54	
56	140, 46	73, 49, 46	107, 54	90, 51, 45
58	89, 51, 46	112, 42	132, 54	
59	192	192	91, 54	
66	140, 46	186	87, 79	
68	192	192	138	
72	110	73, 98	156	
73	195	98	94	
82	97	73	80	

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