CHAPTER III
EXPERIMENT

This chapter presents information regarding chemicals, apparatus and general procedure. The details of each step are described below.

3.1 Chemicals and apparatus

All chemicals were obtained from commercial suppliers (Fluka, Merck or Aldrich Chemical Co., Ltd.,) and used without further purification. TentaGel S RAM Fmoc resin (0.24 mmol/g loading) (Fluka) was used as the solid support for solid phase peptide synthesis. Fmoc-L-Lys(Boc)-OPfp and Fmoc-L-Glu(OtBu)-OH were obtained from Calbiochem Novabiochem Co., Ltd. Fmoc-L-Ser(tBu)-ODbt was obtained from Fluka. The four Fmoc-protected, Pfp-activated pyrrolidinyl PNA monomers (Fmoc-A^{Bz}-OPfp, Fmoc-C^{Bz}-OPfp, Fmoc-G^{Ibu}-OH and Fmoc-T-OPfp), ACPC and APC spacer were synthesized by Dr. Chalotorn Boonlua, Dr. Woraluk Mansawat, Ms. Boonsong Ditmangklo and Mr. Nattapon Maneelun according to previously published protocols [13, 14]. *O*-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) was obtained from Fluka. 1-Hydroxy-7-azabenzotriazole (HOAt) was purchased from GLBiochem (Shanghai, China). Pentafluorophenyl trifluoroacetate was obtained from Aldrich. Anhydrous *N*,*N*-dimethylformamide (DMF) for solid phase peptide synthesis was obtained from RCI Labscan (Thailand) and stored over 4A molecular sieves. Tris(hydroxymethane)aminomethane (Tris) (Molecular biology grade) was obtained from USB Co., Ltd. (USA). Tris-Borate EDTA (10x) was obtained from Fluka. Commercial grade of solvents for column chromatography were distilled before use. HPLC grade MeOH and MeCN for HPLC purification were obtained from Merck and used as received. MilliQ water was obtained from ultrapure water system with Millipak® 40 filter unit 0.22 µm, Millipore (USA) and was filtered through a Nylon membrane filter (13 mm ϕ, 0.45 µm pore size) before HPLC use. Graphite powder (mesh size < 100) was purchased from Sigma-Aldrich. Carbon ink and silver/silver chloride were purchased from Acheson (California, USA). Diethylene glycol monobutyl ether and ethylene glycol monobutyl ether acetate, used as binder solution in the ink preparation step, were purchased from Merck. Poly(quaternized dimethylamino)ethyl methacrylate (PQDMAEMA, M_n = 13,777 Da) and polyacrylic acid (PAA, M_n = 7890 Da) was synthesized and supplied by

Dr. Voravee Hoven (Organic Synthesis Research Unit, Chulalongkorn University). All synthetic oligonucleotides were purchased from Pacific Science (Bangkok, Thailand) or BioDesign Co.,Ltd. (Bangkok, Thailand). Strains of White Spot Syndrome Virus (WSSV), Infectious hypodermal and hematopoietic necrosis virus (IHHNV), Yellow-Head Virus (YHV), and Taura syndrome virus (TSV) were employed in specificity test of LAMP samples. The first two viral specimens were provided by Chachoengsao Coastal Fisheries Research and Development Center, Chachoengsao, Thailand, and the latter two specimens were from TSM Chanisa Hatchery, Phang Nga, Thailand. For the surveillance study, forty-nine field specimens from frozen samples supplied from private farms in Phang Nga province. LAMP-amplified WSSV and other shrimp virus DNA samples were obtained from Dr. Piyasak Chaumpluk (Laboratory of Plant Transgenic Technology and Biosensor, Department of Botany, Faculty of Science, Chulalongkorn University). PCR-amplified Human Papilloma Virus (HPV) DNA samples and 1AQ-GCTGGAGGTGTATG-LysNH_2 (1AQ-HPV-Lys) probe were obtained from Mr. Sakda Jampasa (Program in Petrochemical and Polymer Science, Chulalongkorn University). PCR-amplified Human Leukocyte Antigen (HLA) DNA samples were obtained from Prof. Dr. Nattiya Hirankarn (Department of Microbiology. Faculty of Medicine, Chulalongkorn University).

The progress of the reaction was monitored by thin layer chromatography (TLC) (D.C. silica gel 60 F254 0.2 mm thickness was purchased from Merck) and visualized using UV light (254 nm). NMR spectra were recorded on a Varian Mercury+ 400 or Bruker Avance 400 operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Chemical shifts were referenced to the residual protonated solvent peaks. Reverse phase HPLC experiments were performed on Water Delta 600^{TM} system with gradient mode. Concentration and melting temperature of synthetic oligonucleotides were recorded on a CARY 100 Bio UV-Visible spectrophotometer (Varian Ltd.). MALDI-TOF MS spectra were recorded on a Microflex MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) in linear positive mode using α-cyano-4-hydroxycinnamic acid (CCA) as a matrix. All electrochemical measurements were performed on a PGSTAT 30 potentiostat (Metrohm Siam Company Ltd.), and controlled with the General Purpose Electrochemical System (GPES) software.

3.2 General procedures

3.2.1 Synthesis and characterization of electrochemically-active reporters

Several types of electrochemically-active reporters were evaluated as the redox-active label on acpcPNA including anthraquinone (AQ) and methylene blue (MB). All of them were functionalized with a carboxyl group in order to attach to amino group of acpcPNA via acylation.

3.2.1.1 Synthesis of 4-(anthraquinone-1-yloxy)butyric acid (1AQ) (1)

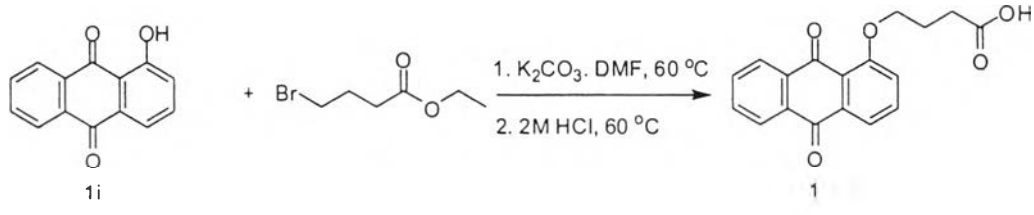

Figure 3.1 Synthesis of 4-(anthraquinone-1-yloxy)butyric acid (1AQ)

1-Hydroxyanthraquinone (1i) (0.10 g, 0.45 mmol, 1.0 equiv.) and potassium carbonate (0.07 g, 0.51 mmol, 1.1 equiv.) were dissolved in 4 mL of DMF. Next, ethyl 4-bromobutyrate (0.11 g, 0.56 mmol, 1.2 equiv.) was added to the solution and the reaction was refluxed for 12 hrs. After reaction was completed as monitored by TLC, the solvent was evaporated and product was purified by column chromatography (EtOAc:Hexanes=10:90) to give ethyl 4-(anthraquinone-1-yloxy)butyrate (1ii) as a yellow solid (0.13 g, 0.38 mmol, 84% yield from 1i). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.25 (t, J = 6.8 Hz, 3H), 2.26 (quintet, J = 6.8 Hz, 2H), 2.73 (t, J = 7.2 Hz, 2H), 4.14 (q, J = 7.2 Hz, 2H), 4.23 (t, J = 5.6 Hz, 2H), 7.33 (dd, J = 8.4, 2.4 Hz, 1H), 7.74 (m, 1H), 7.95 (d, J = 7.6 Hz, 2H), 8.36 (d, J = 7.2 Hz, 1H), 8.23 (m, 2H).

Ethyl 4-(anthraquinone-1-yloxy)butyrate (1ii) (0.13 g, 0.38 mmol, 1.0 equiv.) was refluxed with 2 mL of 2 M HCl (4.0 mmol, 10.5 equiv.) overnight. After the reaction was completed as monitored by TLC, the precipitated product was filtered, washed with water and dried to give compound 1 as a yellow solid (0.12 g, 0.35 mmol, 92% yield from 1ii). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.04 (quintet, J = 6.8 Hz, 2H), 2.60 (t, J = 7.6 Hz, 2H), 4.20 (t, J = 6.0 Hz, 2H), 7.59 (dd, J = 6.8, 2.8 Hz, 1H), 7.87 (m, 4H), 8.15 (m, 2H), 12.17 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 24.2 (CH_2), 30.0 (CH_2), 68.0 (CH_2), 119.0 (CH), 119.9 (C), 120.7 (C), 126.1 (CH), 126.2 (CH), 132.0 (C), 133.5 (CH), 134.6 (CH), 135.0 (C), 135.4 (CH), 159.3 (C-O), 174.2 (C=O), 181.1 (C=O), 182.8 (C=O).

3.2.1.2 Synthesis of 4-(anthraquinone-2-yloxy)butyric acid (2AQ) (2)

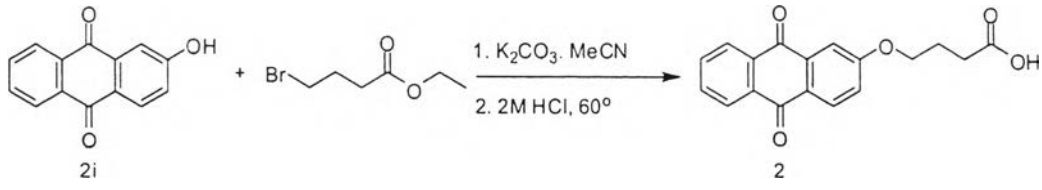

Figure 3.2 Synthesis of 4-(anthraquinone-2-yloxy)butyric acid (2AQ)

2-Hydroxyanthraquinone (2i) (0.54 g, 2.39 mmol, 1.0 equiv.) and potassium carbonate (0.40 g, 2.92 mmol, 1.1 equiv.) were dissolved in 40 mL of MeCN. Next, ethyl 4-bromobutyrate (0.50 g, 2.48 mmol, 1.1 equiv.) was added to the solution and stirred at room temperature for 24 hrs. After reaction completed, monitored by TLC, the solvent was evaporated and the product was purified by column chromatography (EtOAc:Hexanes=10:90) to give ethyl 4-(anthraquinone-2-oxy)butyrate (2ii) as a yellow solid (0.54 g, 1.60 mmol, 66% yield from 2i). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.29 (t, *J* = 7.2 Hz, 3H), 2.21 (quintet, *J* = 6.8 Hz, 2H), 2.57 (t, *J* = 6.8 Hz, 2H), 4.19 (q, *J* = 7.2 Hz, 2H), 4.24 (t, *J* = 6.4 Hz, 2H), 7.28, (dd, *J* = 8.6, 2.6 Hz, 1H), 7.73 (d, *J* = 2.6 Hz, 1H), 7.81(m, 2H), 8.29 (d, *J* = 8.6 Hz, 1H), 8.32 (m, 2H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 14.2 (CH_3), 24.4 (CH_2), 30.6 (CH_2), 60.6 (CH_2-O), 67.6 (CH_2-O), 110.6 (CH), 121.4 (CH), 127.2 (CH), 129.8 (C), 133.6 (C), 133.7 (CH), 134.2 (CH), 135.6 (C), 163.6 (C-O), 172.9 (C=O), 182.1 (C=O), 183.4 (C=O).

Ethyl 2-(anthraquinone-1-yloxy)butyrate (2ii) (0.56 g, 1.66 mmol, 1.0 equiv.) was refluxed with 11 mL of 2 M HCl (22.0 mmol, 13.25 equiv.) for 6 hrs. After the reaction was completed as monitored by TLC, the precipitated product was filtered, washed with water and dried to give compound 2 as a yellow solid (0.43 g, 1.38 mmol, 83% yield from 2ii). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.00 (quintet, *J* = 6.8 Hz, 2H), 2.41 (t, *J* = 7.2 Hz, 2H), 4.21 (t, *J* = 6.4 Hz, 2H), 7.43, (dd, *J* = 8.4, 2.4 Hz, 1H), 7.58 (d, *J* = 2.4 Hz, 1H), 7.91 (m, 2H), 8.17 (m, 3H), 12.19 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 24.0 (CH_2), 30.0 (CH_2), 67.6 (CH_2), 110.7 (CH), 121.0 (CH), 126.3 (C), 126.6 (CH), 126.6 (CH), 129.5 (CH), 133.0 (C), 133.0 (C), 134.1 (CH), 134.5 (CH), 135.0 (C), 163.2 (C-O), 174.0 (C=O), 181.2 (C=O), 182.3 (C=O).

3.2.1.3 Synthesis of methylene blue butyric acid (3)

Methylene blue butyric acid was synthesized according to a literature procedure [68]. Due to the small quantity available, it was only characterized by MALDI-TOF MS: *m/z* (MALDI-TOF) calcd for M·H$^+$ 355.14, found 355.29.

3.2.2 Synthesis of labeled acpcPNA probes

3.2.2.1 Synthesis of acpcPNA probes

The acpcPNA was synthesized by Fmoc-solid phase peptide synthesis method according to our previously published protocol [9]. For internal labeling of the acpcPNA, an ACPC spacer at the specified position was replaced with an APC spacer as described earlier [69]. The synthesis started with deprotection of the Fmoc protecting group on the solid support (1.5 μmol) with 100 μL solution of 2% DBU and 20% piperidine in DMF (100 μL). Then, it was coupled with the PNA monomer or ACPC/APC spacer which was previously activated by treatment with the solution of DIEA (8 equiv) and HOAt (4.0 equiv.) (for Pfp-activated monomers) or HATU (4 equiv.) (for free acid monomers) in DMF (30 μL) for 40 min. Next, capping of unreacted amino groups was performed by treatment with the solution of DIEA (8 equiv.) and acetic anhydride (34.5 equiv.) in DMF (30 μL) for 5 min. This cycle was repeated until the desired PNA sequence was obtained.

3.2.2.2 Labeling electrochemically-active reporter onto acpcPNA via acylation reaction

After the PNA synthesis was completed, it was labeled with the electrochemically active reporter at the internal or terminal position while still on the solid support. Before labeling at the internal position, the free amino group at the end of the PNA strand was acetylated by treatment with acetic anhydride/DIEA under the same capping conditions as described in 2.2.1. The carboxyl-functionalized redox-active reporter was activated by dissolving 7.5 μmol of the acid in a minimum volume of DMF. To this solution was added DIEA (10 μL, 57.4 μmol, 8 equiv.) followed by PfpOTfa (5 μL, 29.1 μmol, 4 equiv.). After reaction completed as monitored by TLC, CH_2Cl_2 was added to the solution. This solution was extracted with 10% HCl followed by saturated aqueous $NaHCO_3$ solution (3 times). The organic phase was dried under a stream of N_2 gas to give a crude Pfp-activated label, which was dissolved in 100 μL of DMF containing DIEA (20 μL, 114.8 μmol, 16 equiv.) before reacting with the PNA on the solid support overnight at room temperature. The progress of the reaction was monitored by MALDI-TOF mass spectrometry after cleavage of some PNA samples from the resin by treatment with trifluoroacetic acid (TFA). For mixed base sequence, the benzoyl (Bz) group on A and C, isobutyryl (Ibu) group on G and trifluoroacetyl (Tfa) group on APC spacer were simultaneously removed by treatment with aqueous ammonia/dioxane 1:1 in a sealed tube at 60 $^{\circ}C$

overnight prior to the cleavage from the resin. The PNA was cleaved from the solid support by treatment with TFA (3 x 100 μL x 1 hr) and the combined TFA cleavage mixture was evaporated under a stream of N_2 gas. The residue was washed with diethyl ether 3 times and dried at room temperature before preparing to be crude PNA solution.

The probe sequences were designed to have only thymine base in the test sequences (T2 and T9), and partial sequences of WSSV, HPV, HLA B1502 and B1513 as shown in **Table 1.**

Table 3.1 PNA probe sequences used in this work

Code	Sequence (N→C)
1AQ-T2-Lys	1AQ-TT-LysNH_2
2AQ-T2-Lys	2AQ-TT-LysNH_2
MB-T2-Lys	MB-TT-LysNH_2
1AQ-T9-Lys	1AQ-TTT TTT TTT-LysNH_2
2AQ-T9-Lys	2AQ-TTT TTT TTT-LysNH_2
MB-T9-Lys	MB-TTT TTT TTT-LysNH_2
T4-(1AQ)-T5-Lys	TTTT-(1AQ)-TTTTT-LysNH_2
T4-(2AQ)-T5-Lys	TTTT-(2AQ)-TTTTT-LysNH_2
T4-(MB)-T5-Lys	TTTT-(MB)-TTTTT-LysNH_2
2AQ-WSSV-Lys	2AQ-TCA AAT TCA GA-LysNH_2
2AQ-WSSV-Glu	2AQ-TCA AAT TCA GA-GluNH_2
2AQ-WSSV-Ser	2AQ-TCA AAT TCA GA-SerNH_2
2AQ-B1502-Lys	2AQ-CGCGCAGGTTCC-LysNH_2
2AQ-B1513-Lys	2AQ-GGAGCGCGATCC-LysNH_2

3.2.2.3 Purification and characterization of labeled acpcPNA probes

The crude PNA sample was dissolved in 120 µL MilliQ water. The solution was filtered through a nylon membrane filter (0.45 µm) before being purified by reverse phase HPLC performing on Water Delta 600™ system on an ACE 5 C18-AR (150 x 4.6 mm) HPLC column, eluting with a gradient system of 0.1% TFA in methanol/water. The HPLC gradient system consisted of solvent A (0.1% TFA in MilliQ water) and solvent B (0.1% TFA in methanol). The elution started with A:B (90:10) at flow rate 0.5 mL/min for 5 min followed by a linear gradient to A:B (10:90) over a period of 70 min, then holding for 10 min before reverting back to A:B (90:10). The collected fractions (monitored with UV detection at 260 nm) were analyzed by MALDI-TOF MS. Fractions containing only the desired products were combined and the solvents removed by freeze-drying to afford the purified PNA.

3.2.2.4 Determination of PNA concentration

The concentration of labeled acpcPNA was determined by UV spectrophotometry on a CARY 100 Bio UV-Visible spectrophotometer (Varian, Inc., USA) at 25 °C with baseline subtraction. The labeled acpcPNA 2 µL was added to a 1000 µL solution of 10 mM sodium phosphate buffer pH 7.0 in MilliQ water in a 10 mm path length quartz cuvette. The concentration was calculated from molar extinction coefficient at 260 nm (ε_{260}) of the unlabeled PNA according to the web-based software developed in-house [70]. Molar extinction coefficients of the label were determined spectrophotometrically. The 1 mM stock solution of 1-hydroxyanthraquinone, 2-hydroxyanthraquinone and methylene blue were prepared and diluted to 5, 10, 15, 20, 25 and 30 µM for absorbance measurement. The slope of the plot between concentration and absorbance at 260 nM was the molar extinction coefficient of the label (ε_{260} (MB) = 6.6 mL·µmol^{-1}·cm^{-1}, ε_{260} (1AQ) = 27.1 mL µmol^{-1}·cm^{-1}, ε_{260} (2AQ) = 22.7 mL µmol^{-1}·cm^{-1}) (see Figure A6).

3.2.2.5 PNA-DNA binding properties

Hybridization property of the labeled acpcPNA were studied and compared with unlabeled acpcPNA. The T_m measurements were performed on 260 nm on a CARY 100 Bio UV-Visible spectrophotometer (Varian Ltd.) equipped with a thermal melt system by scanning and recording A260 in heating from 20 °C to 95 °C (heating block temperature) with a temperature ramp of 1 °C/min and collect absorbance of solution at 260 nm. The sample for the T_m measurement was prepared by mixing

oligonucleotide and PNA solution to give the final concentration of 1 μM in 10 mM sodium phosphate buffer (pH 7.0) in 1000 μL Milli-Q water in a 10 mm quartz cell. The samples were equilibrated at the starting temperature for 10 min and cooled down for 10 min after finished collect data. Melting temperature (T_m) was calculated from the maximum of the first derivative of normalized absorbance at 260 nm as a function of corrected temperature after smoothing using KaliedaGraph 3.6 (Synergy Software). T_m values obtained from independent experiments were accurate to within ± 0.5 °C. Correct temperature and normalized absorbance are defined as follows.

Correct Temp. = $(0.9696 \times T_{block}) - 0.6068$

Normalized Abs. = Abs_{obs}/Abs_{init}

The equation for temperature correction was previously obtained by measuring the actual temperature in the cuvette using a temperatutre probe and plotting against the set temperature (T_{block}) from 20-95 °C. A linear relationship $Y = 0.978X - 0.6068$ and $R^2 > 0.99$ was obtained [71].

3.2.3 Preparation of samples and buffers

3.2.3.1 Preparation of stock buffer solution used in study of buffer effect

Citrate buffer solution

Citric acid ($C_6H_8O.7H_2O$) (5.55 g, 25.0 mmol) and trisodium citrate ($Na_3C_6H_5O_7.2H_2O$) (7.35 g, 25.0 mmol) were dissolved separately in water (25 mL each) to give solutions containing 1.0 M citric acid and 1.0 M sodium citrate, respectively. Then, 5.75 mL of 1.0 M citric acid and 6.75 mL of 1.0 M sodium citrate were mixed together and the pH was adjusted to 4.6 with conc. hydrochloric acid or 6 M sodium hydroxide.

MES buffer solution

2-Morpholinoethanesulfonic acid ($C_6H_{13}NO_4S$) (0.98 g, 5.0 mmol) was dissolved in 5 mL of water to give 1.0 M MES. The pH was adjusted to 7.2 with conc. hydrochloric acid or 6 M sodium hydroxide.

Tris-HCl buffer solution

Tris(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$) (3.03 g, 25.0 mmol) and conc. HCl (2.1 mL, 25.2 mmol) were dissolved separately in water (25 mL) to give 1.0 M Tris-HCl and 1.0 M HCl, respectively. Next, 8.30 mL of 1.0 M Tris-HCl and 4.87 mL of

1.0 M HCl were mixed together and the pH was adjusted to 7.4 or 8.0 with conc. hydrochloric acid or 6 M sodium hydroxide.

Tris-Borate buffer

A commercial stock solution of Tris-Borate-EDTA (10x) (Fluka, consisting of 1.3 M tris(hydroxymethyl)aminomethane, 450 mM boric acid and 25 mM EDTA.Na_2 in H_2O) (10 mL) was diluted with 3 mL of water to give a 1.0 M Tris-Borate stock solution. The pH was adjusted to 9.0 with conc. hydrochloric acid or 6 M sodium hydroxide.

3.2.3.2 DNA amplification by Loop-Mediated Isothermal Amplification (LAMP)

The LAMP samples were provided by Dr. Piyasak Chaumpluk (Laboratory of Plant Transgenic Technology and Biosensor, Department of Botany, Faculty of Science, Chulalongkorn University) and were used as received. In brief, the four primers used in this WSSV sequence were WSSV F3, WSSV B3, WSSV FIP, and WSSV BIP (see **Table 2**). The reaction mixture consists of 40 mM Tris pH 8.8, 20 mM KCl, 16 mM $MgSO_4$, 0.2% Tween 20, 1.6 M betaine, 2.8 mM dNTP, and 8 U Bst DNA polymerase and was incubated at 63 $^{\circ}C$ for 30 min. The successful formation of the LAMP products was verified by standard 3% agarose gel electrophoresis after staining with ethidium bromide and visualized under a UV transilluminator.

Table 3.2 Primer sequences used in LAMP amplification of WSSV samples (Chaumpluk, P., personal communication)

Primer name	Primer Sequence (5' to 3')	Genomic position*
WSSV F3	ATC AAA AAA ATT GGA GAC TTG G	255368-255381
WSSV B3	CAT CAG AGG AAT AAC CTA CAT TC	255558-255580
WSSV FIP	GAC TGC CAA ACA AAA TAA GGC TTT TCA GAG TTT CTT CTT CCA TCG	255401-255418, 255448-255471
WSSV BIP	TGG GTT GGG GAT GAA CAT ACC AAT AAT AGG TGC AGA AGT AAA AGG	255472-255492, 255529-255549

*With reference to sequence accession No. JX515788

JX515788

255361 tttcttgatc aaaaaaattg gagacttggt atcatgagca gagtttcttc ttccatcgcc

255421 aactttaaaa ttgaacaaga gtcatcaaaa gccttatttt gtttggcagt ctgggttggg

255481 gatgaacata cccctaaatt cagacttagt gtatggaaga actggaagcc ttttacttct

255541 gcacctatta ttgtccagaa tgtaggttat tcctctgatg ttttctggca tgaaactctt

255601 agaagcaaaa ttgttgatcg gtcaagggac ctgatagaaa caaaagtgac aaagaaaatt

3.2.3.3 DNA amplification by PCR

The DNA samples from HPV-positive and negative cell lines were amplified by PCR under supervision of Dr. Pattamawadee Yanatatsaneejit (Human Genetics Research Group, Department of Botany, Faculty of Science, Chulalongkorn University). The PCR amplification of HPV type 16 required two primers (forward and reverse, see **Table 3**). The reaction mixture consisted of 0.4 µM forward and reverse primers, 50 ng/µL Cell-line (SiHa) HPV type 16, 0.2 mM dNTPs, buffer 10x, 15 mM $MgCl_2$ and 0.5 unit/µL Taq polymerase. The PCR was performed for 35 cycles involving denaturation (95 °C, 10 min), primer annealing (95 °C, 30 sec to 52 sec, 30 sec), followed by extension of the annealed primers (72 °C, 30 sec) and final elongation (72 °C, 7 min).

The PCR products were analyzed by 2% agarose gel electrophoresis after staining with ethidium bromide and visualized under a UV transilluminator.

Table 3.3 Primer sequence used in PCR amplification [26]

Primer name	Primer Sequence (5' to 3')
Forward primer	CACTATTTTGGAGGACTGGA
Reverse primer	GCCTTAAATCCTGCTTGTAG

3.2.3.4 Denaturation of duplex DNA (dsDNA) samples before electrochemical measurement

Two approaches were used to denature dsDNA samples: thermal or chemical denaturations. In the thermal method (for LAMP samples), the PNA sample was prepared from the stock solution to give the final concentration of 50 nM in 10 mM Tris-HCl buffer (pH 8.0) (100 µL). After that, 1 µL of LAMP sample was added to the solution. The mixture was heated to 90 °C for 10 min and slowly cooled down to room temperature. Alternatively, 1 µL of the LAMP sample was prepared in 100 µL of 10 mM Tris-HCl buffer (pH 8.0) and then heated to 90 °C for 10 min and rapidly cooled down at 0 °C. The PNA was immediately added to give the final concentration of 50 nM PNA in 100 µL of 10 mM Tris-HCl buffer (pH 8.0).

For the chemical denaturation method (for PCR samples), 5 µL of the PCR sample was treated with 10 µL of 1.0 M NaOH for 5 min and then neutralized with 10 µL of 3 M NaOAc (pH 5.0). The PNA was added to give the final concentration of 50 nM PNA in 100 µL of 10 mM Tris-HCl buffer (pH 8.0).

3.2.4 Preparation of electrodes and electrochemical measurements

3.2.4.1 Preparation of screen printed carbon electrode (SPCE)

The electrode was prepared as previously described [26]. The screen-printed block was made by Chaiyaboon Co. Ltd. (Bangkok, Thailand). Diethylene glycol monobutyl ether and ethylene glycol monobutyl ether acetate (ratio 1:1) used as binder solution in the carbon ink preparation step and nail polish was used as insulator. PVA was used as screen substrate. The SPCE electrode consists of Ag/AgCl (reference electrode and connecting pads), carbon (working and counter electrode) and insulator. The Ag/AgCl was screened in the first step (2 times) followed by carbon ink (2 times) and insulator (only once) on polyvinyl chloride (PVC). After each screening step, the electrode was dried at 55 °C for 1 hr.

Positively charged and negatively charged modified screen printed carbon electrode were prepared similarly to the unmodified SPCE explained above, but with the appropriate polymer added to the carbon ink mixture. The polymer was prepared to have desired concentration and mixed with carbon ink in a ratio of carbon ink, graphite powder, polymer solution and binder solution (1 g: 0.2 g: 0.35 g: 1 mL).

3.2.4.2 Electrochemical measurements

Electrochemical experiments were performed on PCSTAT 30 potentiostat (Metrohm Siam Company Ltd., Switzerland) and controlled with the General Purpose Electrochemical System (GPES) software version 4.9 (Econ Chemie B.V., Utrecht, The Netherlands) at Electrochemistry and Optical Spectroscopy Research Unit, Department of Chemistry, Faculty of Science, Chulalongkorn University. The sample was prepared by mixing calculated amount of stock DNA (duplex DNAs required prior denaturation, see 3.2.3.4) and PNA solution together to give the desired final concentration in 10 mM Tris-HCl buffer (pH 8.0) (or other appropriate buffers for studying the buffer effect) and the final volume was adjusted to 100 µL. The sample solution (20 µL) was dropped onto the electrode covering working, counter and reference electrode before the signal was measured by square-wave voltammetry (SWV) (unoptimized parameters: frequency 30 Hz, step potential 0.01 V and amplitude 0.05 V). The electrochemical signal was obtained from the peak height after baseline subtraction.

Moreover, the mechanism of electron transfer on each kind of electrode was studied by measuring the signal of $[Fe(CN)_6]^{3-/4-}$ (1 mM) in 0.5 mM KCl (20 µL) at different scan rates by cyclic voltammetry (CV).