CHAPTER IV

MATERIALS AND METHODS

Part I. Development of multiplex PCR for detection of HBV DNA and HCV RNA

1. Chemical reagents and instruments

Most of the chemical agents were molecular biology grade and were purchased from Sigma, USA. NucliSens Isolation kit and Lysis buffer were purchased from Oganon Teknica Boxtel , NL. Enzymes used in molecular technique were purchased from Promega, USA. Name lists of all chemical reagents and instruments were shown in the appendices.

2. Oligonucleotide primers

Oligonucleotide primers were synthesized with a DNA synthesizer (Gene Assembler Special, Pharmacia, LKB). Two sets of HCV primers were generated from the 5′ non-coding region of the published sequences (72), which is highly conserved among HCV isolates. The external primers were sense, nucleotide positions 18 to 37, 5′-GGCGACACTCCACCATGAAT-3′ (primer TJC1), and antisense, nucleotide positions 289 to 344, 5′-TCGCAAGCA CCCTATCAGGCA -3′ (Primer TJC 4). The internal primers were as follows: sense, nucleotide positions 51 to 69, 5′-GGAACTACTGTCTTCACGC-3′(primer TJC 3) and antisense, nucleotide positions 170 to 190, 5′-GTTTATCCAAGAAAGGACCCG-3′(primer HCV 2).

Two sets of HBV primers were generated from the region of the published sequences (73), which is highly conserved among HBV isolates. The external primers were sense, nucleotide positions 1766 to 1788, 5′-CTTTGTACTAGGAGGCTGTAGGC-3′ (primer TJB 1), and antisense, nucleotide positions 2267 to 2287, 5′-GGAGGAGTGCGAATCCACACT-3′ (Primer TJB 2). The internal primers included: sense, nucleotide positions 1891 to 1912, 5′-GCTTTGGGGCATGGACATTGAC-3′(primer TJB 3) and antisense, nucleotide positions 2135

to 2156 , 5′-CTACTAATTCCCTGGATGCTGG-3′(primer TJB4). The HBV and HCV primers were designed to amplify at the same annealing temperatures and generated a different PCR length. The nucleotide sequences of primers are shown in Table 3.

Table 3. The nucleotide sequences of HBV and HCV primers

Primer	Sequence 5′→3′	Polarity	Nucleotide Position*	PCR Product
HCV Primer				
External				
Primer TJC1	GGCGACACTCCACCATGAAT	Sense	18-37	
Primer TJC4	TCGCAAGCACCCTATCAGGCA	Anti-sense	289-310	292
Internal				
Primer TJC3	GGAACTACTGTCTTCACGC	Sense	51-69	
Primer HCV2	GTTTATCCAAGAAAGGACCCG	Anti-sense	170-190	139
HBV Primer				
External				
Primer TJB1	CTTTGTACTAGGAGGCTGTAGGC	Sense	1766-1788	
Primer TJB2	GGAGGAGTGCGAATCCACACT	Anti-sense	2267-2287	524
Internal				
Primer TJB3	GCTTTGGGGCATGGACATTGAC	Sense	1896-1912	
Primer TJB4	CTACTAATTCCCTGGATGCTGG	Anti-sense	2135-2156	266

*Accession numbers M 67463 for HCV and D 12980 for HBV

3. Pools of blood donations

Blood donor samples were collected into a sterile tube as an extra sample from healthy repeated blood donors (negative for HBsAg, anti-HCV, anti-HIV, HIV-Ag and syphilis) in 7-ml sterile tubes (National Blood Center, Thai Red Cross Society). Serum was separated by centifugation at 2,500 g for 10 minutes at room temperature. Pools of 20 blood donations were assembled by using 100 µl each individual serum, and then aliquots of 2-mL pools were stored at -70°c until being thawed for analysis.

The HBV DNA positive serum obtained from HBV-infected patient (HBsAg positive, HBV DNA positive, HCV RT-PCR negative), HCV RNA positive serum obtained from HCV patient (anti-HCV positive, HCV RNA positive and HBV DNA negative). HBV and HCV mixed sera was a mixture (1:1, vol:vol) of HBV and HCV positive serum. Negative control was plasma from healthy donors (HBV DNA and HCV RNA negative). Both DNA and RNA were extracted simultaneous from these samples using a nucleic acid isolation kit (NucliSens, Organon Teknika, Boxtel, NL) and amplified with the Multiplex HBV/HCV PCR. Detection of amplification products was performed by gel electrophoresis.

4. Extraction of nucleic acid

Nucleic acid extraction was performed by Boom method (74). This method used a nucleic acid isolation kit (NucliSens, Organon Teknika, Boxtel, NL) as described. The washing buffer and lysis buffer were warmed at 37 °C for 30 minutes. Nine ml of Lysis buffer and 2 ml of pooled unit sera were added in tube and spun at 1500 g for 2 minutes. Then, added 50 µl silica suspension were added into each tube for 10 minutes at room temperature (15-30°C). Lysis buffer tubes was regularly inverted every two minutes to prevent silica from setting on the bottom. Then they were spun at 1,500 g for 2 minutes. The supernatant was removed by 10 ml plastic pipette, leaving about 0.5 ml residual fluid in the tube. The 0.5 ml residual was removed with 1,000 µl disposable tip pipette, using a fresh sterile tip; without whirling up the pellet. One ml of wash buffer was added into the lysis buffer tube and then the pellets were mixed. The content of lysis buffer tube was transferred to a fresh 1.5 ml microcentrifuge tube. After

centrifugation at 10,000 g for 30 seconds, the supernatant was removed. The silica pellet was washed again with 1 ml of washing buffer, twice with 1 ml of 70% ethanol, and once with 1 ml of acetone. After centrifugation and the supernatant were discarded, the silica pellets was dried at 56°C for 10 minutes in heating block. Finally, 50 µl elution buffer were added and suspended in the tube. The tube was warmed at 56°C for 10 minutes to elute the nucleic acids and vortexed after 5 minutes to prevent silica from setting on the bottom. The tube was centrifuged for 2 minutes at 10,000 g. The supernatant nucleic acids were then transferred to a fresh test tube and stored at –70 °C until the time for analysis.

5. PCR Amplification

A multiplex PCR was designed for simultaneous detection of HBV DNA and HCV RNA. The DNA and RNA extracted from serum were used as templates. The reactions were performed in a volume of 50 µl containing 40 µl of reaction mixture (5 µl of 10x buffer for *Taq* polymerase: 50 mM KCl, 100 mM Tris-HCl pH 9.0 at 25 °C, and 1 % Triton X –100, 4 µl of 25 mM $MgCl_2$, 4 µl of 2.5 mM dNTP, 1 µl of each 20 pmole/µl primer TJC1 and primer TJC4, 0.5 µl of each 25 pmol /µl primer TJB1 and primer TJB 2, 0.5 µl of 40 U/ µl RNase inhibitor, 0.5 µl of 200 U/µl M-MLV reverse transcriptase , 0.5 µl of 5 U/ µl *Taq* polymerase, and 22.5 µl of DEPC-treated water) and 10 µl of viral templates. This mixture was covered with 2 drops of mineral oil. The reverse transcription was performed in a Gene Amp 480 thermocycle (Perkin-Elmer Cetus) with one cycle at 37°C for 30 minutes, and 95°C for 3 minutes. The first PCR amplification step was performed for 30 cycles as follows: DNA denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension at 72°C for 5 minutes was performed in the last cycle.

Nested PCR was carried out under mineral oil in a PCR reaction mixture in a volume 50 µl containing 5 µl of 10X buffer, 3 µl of 25 mM $MgCl_2$, 4 µl of 2.5 mM dNTP, 1 µl of each 20 pmol/µl primer TJC 3 and primer HCV 2 , 0.5 µl of each 25 pmol/µl primer TJB 3 and primer TJB 4, 0.5 µl of 5 U/µl *Taq* polymerase and 32.5 µl of DEPC-treated water. Two

microliters of the first PCR reaction were transferred to the reaction mixture. The nested PCR was performed for 30 cycles using the programme as same as the first PCR-amplification.

6. Detection of amplification product.

Ten microliters of nested PCR product were analyzed by electrophoresis on a 1.5% agarose gel, consisted of 50 μg/ml ethidium bromide in a Tris-borate EDTA buffer (pH 8.0). The electrophoresis was carried out at 100 volts for 30 minutes. The PCR products were visualized under UV and a photography was taken by a polaroid camera.

Part II. The limit of detection of multiplex HBV/HCV PCR

The limit of detection of the multiplex PCR for detection of HBV DNA and HCV RNA in 2-mL pooles was evaluated. An aliquot of HBV serum containing approximately 196,294 copies/ml (Amplicor HBV monitor assay, Roche molecular Systems, Branchburg, NJ) and HCV serum containing approximately 360,480 copies/ml (Amplicor HCV monitor assay, Roche molecular Systems, Branchburg, NJ) were kindly provided by Molecular Diagnostic laboratoy, Bangkok General Hospital and were used as quantitative HBV DNA and HCV RNA standards. The standard sera were diluted with negative control (donated plasma, negative for HBsAg, anti-HCV, anti-HIV, HIV-Ag and syphilis). Negative control was also determined and confirmed of negative for HBV DNA PCR and HCV RNA PCR using commercial assays (Roche molecular Systems, Branchburg, NJ). The HBV DNA serum dilutions for sensitivity study were undiluted, 1:10, 1:100, 1:200, 1:400 and 1:1,000. As a result the HBV DNA concentrations were 196,294, 19,629, 1,962, 981, 490, 196 copies/ml (3,925, 392, 39, 19, 9 and 3 copies/assay), respectively. The HCV RNA serum dilutions for sensitivity study were undiluted, 1:10, 1:20, 1:40, 1:80 and 1:800. As a result the HCV RNA concentration were 360,480, 36,084, 18,024, 9,012, 4,506, 450 copies/ml (7,209, 720, 360, 180, 90, and 9 copies/assay), respectively. The concentration of DNA or RNA was calculated from known standard serum, dilution factor, volume of standard serum using for extraction (100 ul) and volume of DNA or RNA using for PCR (10 ul). Dilutions of HBV or HCV positive serum with known DNA or RNA copy numbers were added to the pools to

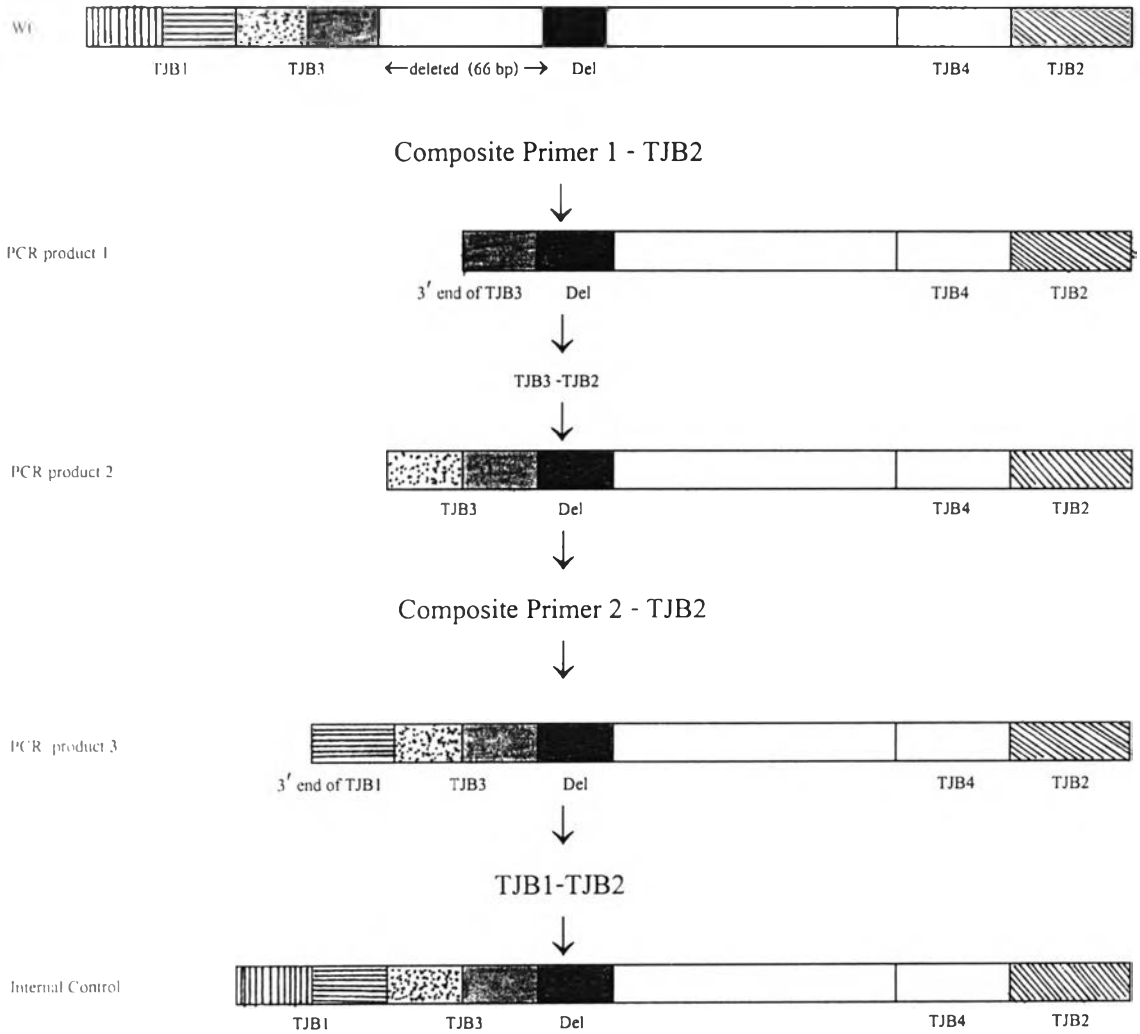
assess the limit detection of the PCR-based pooling assay. The pooling of controls were extracted using a nucleic acid isolation kit; 10 replicate extractions were performed for each diluted sample and a single amplification was performed for each extract. All of the control extracts were amplified with the multiplex HBV/HCV PCR method.

Part III. Construction of the internal control template for nested primer PCR of HBV DNA

1. Synthesis of the HBV PCR internal control template oligonucleotides (19,16,21)

1.1 The internal oligonucleotide control template was designed as shown in Fig. 9, which depicts synthesis of control template and oligonucleotides used. A DNA fragment with a deleted 66-bp sequence of HBV precore region was constructed by amplification of proviral DNA with composite primer 1, CPHBV 1 (containing 14 bases from 3′ TJB 3 sequence and a 15-bp sequence in the region termed Del which located 18 bp 3′ to TJB 3 sequence) and primer 2. Then the PCR products were detected by electrophoresis. The PCR products were 325 bp and were purified by QIA quick PCR purification kit (QIAGEN, Valencia CA, USA).

1.2. Amplification of the resulting fragment with primer TJB 3 and TJB 2 yields 333-bp products (PCR product 2). The PCR product 2 was purified using QIA quick PCR purification kit. The 333-bp construct was amplified with composite primer 2, CPHBV 2 (containing 13 bases from 3′TJB 1 and 14 base from 5′of TJB 3) and primer TJB 2. The amplification yielded a 346-bp construct (PCR product 3) and was purified with QIAquick PCR purification kit. The PCR product 3 was amplified with primer TJB 1 and primer TJB 2. The amplification product yielded 356-bp (internal control). The 356-bp was amplified again with the same primer pair and was purified with QIAquick PCR purification kit. The concentration of the internal control was determined by measurement of optic density at 260 nm and calculated for the copy number to use in this study. The copy number of internal control product was 5.07×10^9 copies/μl.



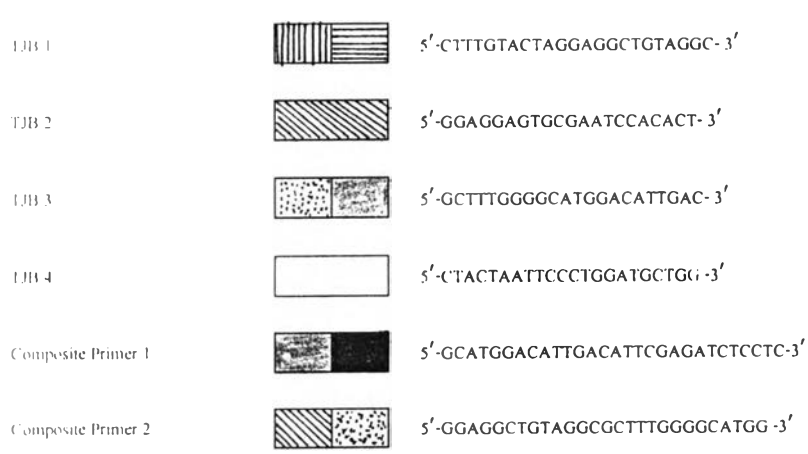


Figure 9 Schemeatic diagrame for the construcion of internal control template and oligonucleotides used.

2. Amplification of HBV DNA by nested PCR.

The first round of PCR amplification was performed in a total volume of 50 μl reaction mixture. Ten μl DNA template were added into 40 μl of reaction mixture containing 5 μl of 10X buffer for *Taq* polymerase (50 Mm KCl, 100 mM Tris-HCl pH 9.0 at 25 ^{o}C, and 1 % Triton X – 100), 3 μl of 2.5 mM $MgCl_2$, 3 μl of 2.5 mM dNTP, 0.5 μl of each 25 pmol/μl primer TJB 1 and primer TJB 2 , 0.5 μl of 5 U/μl *Taq* polymerase and 27.5 μl of water. This mixture was covered with 2 drops of mineral oil. The thermocycle was programmed to denature samples at 94^{o}C for 3 minutes and to amplify through 30 cycles with each cycle consisting of 94^{o}C for 30 seconds (denaturation), 55^{o}C for 30 seconds (primer annealing), 72^{o}C for 30 seconds (extension) and a final extension step at 72^{o}C for 5 minutes.

The nested PCR was carried out under mineral oil in a 50 μl of reaction mixture containing 5 μl of 10X buffer, 3 μl of 2.5 mM $MgCl_2$, 3 μl of 2.5 mM dNTP, 0.5 μl of each 25 pmol /μl primer TJB 3 and primer TJB 4 , 0.5 μl of 5U/μl *Taq* polymerase and 35.5 μl of water. Two microliters of the first PCR reaction were transferred to the reaction mixture. The nested PCR was performed for 30 cycles in the thermocycle, programmed as for the first PCR amplification. As the internal control shares the primer recognition sequences with the HBV wild-type DNA fragment, this procedure yields PCR products of 226 bp length of wild-type HBV and 200 bp length of internal control.

3. PCR purification

The PCR product was purified by QIAquick PCR purification kit (QIAGEN, USA). The method is subsequently described. Five volume of buffer PB were added to 1 volume of the PCR reaction and were mixed. A QIAquick spin column was placed in a provided 2-ml collection tube. The sample was applied to the QIAquick column and centrifuged 30-60 seconds, DNA was adsorbed to the silica-membrane. The contaminants were passed through the column, and the flow-through was discarded. QIAquick colume was placed back into the same tube. Impurities

were washed away, 0.75 ml buffer PE was added to column and centrifuged 30-60 seconds. The contaminants were passed through the column, and the flow-through was discarded. QIAquick colume was placed back into the same tube. The column was centrifuged for 1 minutes at maximum speed. QIAquick colume was placed in a clean 1.5 ml microfuge tube. DNA was eluted with 50 µl EB buffer (10 mM Tris-Cl, pH 8.5). EB buffer was added into the center of the column and centrifuged for 1 minutes. DNA was stored at $-20^{\circ}C$ until it was used.

4. Optimal copy numbers of the internal control for HBV-DNA PCR

The internal control was evaluated for the optimal copy numbers for the PCR reaction. Different dilutions of wide range of internal control (1:10, 1:100, 1:1,000 which equivalent to 250, 25, 2 copies respectively) were mixed with different copy number of wild-type HBV template (392, 196, 39, 3 copies) 1:1, in volume. A negative control (PCR reagent alone) was always included in each run of nested PCR. Wild type template was HBV patient serum which had HBsAg positive and HBV DNA PCR positive.