CHAPTER IV

RESULTS

1. Study subjects

Twenty HIV-1 serodiscordant couples enrolled for this study were from Sunpatong and Doisaket Hospital in Chiang Mai, the northern province of Thailand, during 2001 to 2002. Nineteen couples were from Sunpatong hospital and one was from Doisaket hospital. The HIV-1 seronegative spouses of the HIV-1 seropositive individuals (codes H01-H20) were identified as HEPS as the criteria described in chapter III. Sixteen (80%) of those HEPS were female and 4 (20%) were male. The median of ages (n = 20) was 39 years (range = 20–55). During study, peripheral blood was drawn from each of those seronegative subjects on the first visit and every 3 months follow-up for 1 year to test for the presence of HIV-1 DNA and antibody. All subjects have confirmed HIV-1/2 seronegative by ELISA and lacking of HIV-1 DNA by PCR in every sample collected during the study. The median of CD4+ lymphocyte counts of the HEPS group (n = 20) was 754 cells/µl (39.5%). Twenty HIV-1 seropositive-matched partners (codes P01-P20) were enrolled as positive control, but 2 of them (P05 and P17) died before the time of the blood collection. In this group, 4 (22.22%) were female and 14 (77.77%) were male. The median of ages (n = 18) was 36 years (range = 24–47). All HIV-1 seropositive subjects were confirmed infection as tested by ELISA and Western blot. The median of CD4+ lymphocyte counts (n = 15) was 172 cells/µl (11%). The HIV-1 non-infected group (codes N01-N10) enrolled as the HIV-1 negative control was randomly selected from Thai people with low risk for HIV-1 infection. All of them showed HIV-1/2 seronegative by ELISA and lacking of HIV-1 DNA by PCR test. Six (60%) of those were female and 4 (40%) were male. The median of ages (n = 10) was 24 years (range = 23–48). The median of CD4+ lymphocyte counts (n = 10) was 721 cells/µl (37%). Among these individuals, none had a history of using drug injecting nor STD infections.

The characteristics and the laboratory studies of the HIV-1 serodiscordant subjects and the control subjects are shown in Table 4.

Table 4 Characteristics and laboratory studies of the HIV-1 serodiscordant subjects and the normal control subjects in the study

Parameters	HIV-1 serodiscordant group		Normal control group
	seronegative subjects (n = 20)	seropositive subjects (n = 18)	seronegative subjects (n = 10)
Codes	H01-H20	P01-P20	N01-N10
Age (years): median (range)	39 (20–55)	36 (24–47)	24 (23-48)
Sex: female/male (percent)	16/4 (80/20)	4/14 (22.22/77.77)	6/4 (60/40)
Opportunistic infection (OI) during study (no. positive/n)	NF	10/18	NF
Antiretroviral drugs use during study (no. positive/n)	NF	7/18	NF
HIV-1/2 ELISA (no. positive/n)	0/20	18/18	0/10
HIV *gag* or *pol* DNA (no. positive/n)	0/20	18/18	0/10
CD4+ cells/μl: median (range) [1]	754 (302-1380)	172 (8-873)	721 (633-1338)
CD4+ cell percent: median (range) [1]	39.5 (20-58)	11 (1-29)	37 (22-47)
HIV RNA load (range: copies/ml)[2]	ND	<400 - >750,000	ND

Abbreviations: ND, not determine; NF, not found

[1] CD4+ T cell count at the same visit (visit 4) of CCR5 protein density determination (Dettrairat S., personal communication)

[2] HIV-1 RNA load at visit 4 (Leechanachai P., personal communication)

2. Determination of nucleotide polymorphisms in the promoter region of the CCR5 gene

Genomic DNA samples from the HIV-1 serodiscordant couples (20 HEPS and 18 HIV-1 seropositive spouses) were determined for the nucleotide polymorphisms of the CCR5 promoter region.

2.1 Amplification of the CCR5 promoter region by PCR technique

DNA extracts from 20 HEPS (H01-H20) and 18 HIV-1 seropositive spouses (P01-P20) were amplified for CCR5 promoter/enhancer region corresponding to the nucleotide position 58,548 to 59,737 of CCR5 gene accession number U95626 reported in GenBank. All samples except the samples number H01, P01 and P06 could be readily amplified and produced amplified product of approximately 1,190 bp fragments (Figure 9). The DNA extracts from samples number H01, P01 and P06 were not sufficient for this experiment. All PCR negative controls (H_2O) were absolutely negative with those primers indicating the absence of contamination in this experiment.

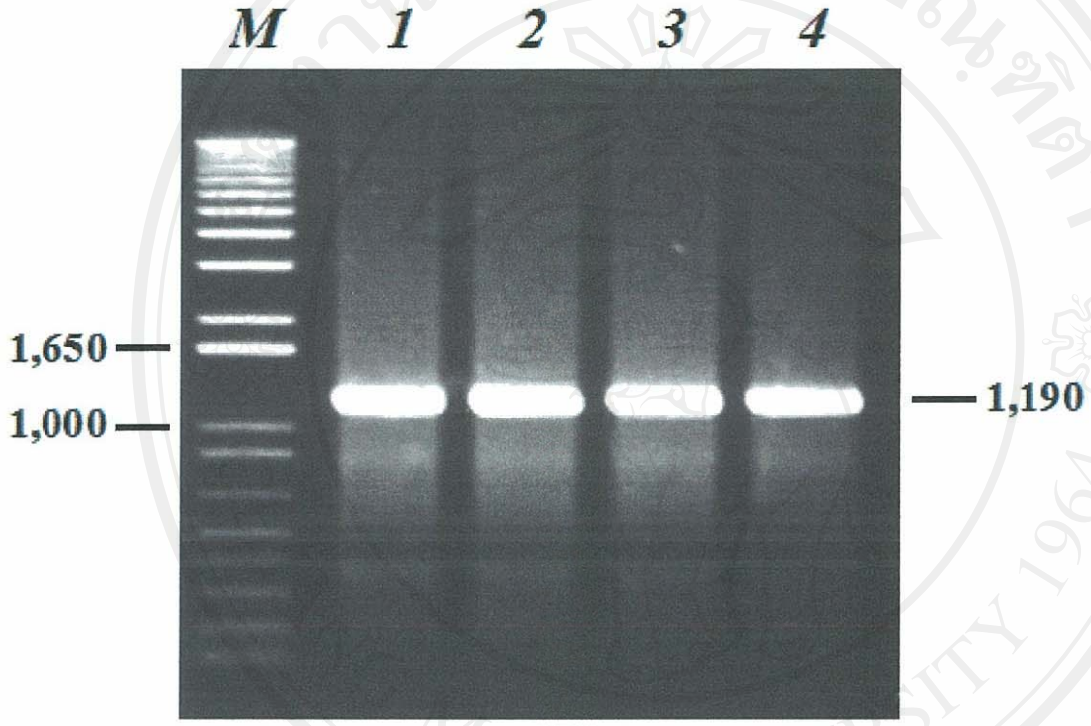

Figure 9 Illustration of the PCR amplification products of the promoter/enhancer region of the CCR5 gene using R5-PF and R5-PR primers. A 1 Kb Plus DNA Ladder was used as a marker (Lane *M*). Lane *1* and *2* show the PCR product of H02 and H03. Lane *3* and *4* show the PCR product of P02 and P03.

2.2 Cloning of the CCR5 promoter/enhancer region

The amplified products of CCR5 promoter region as shown in Figure 9 was purified and cloned into the pGEM®-T Easy Vector using a molar ratio between DNA insert and the vector equal to 3:1. After transformation, the bacterial colonies were first screened for the presence of recombinant plasmid by minipreparation of DNA extract and running on the 1% agarose gel electrophoresis. The plasmid vector containing inserted DNA fragment was differentiated from the empty plasmid by size differences. The recombinant plasmids will increase their sizes to approximately 4.2 kb, while the empty one was 3.0 kb (Figure 10). Only the colonies containing 4.2 kb plasmid were confirmed by using PCR with the R5-PF and R5-PR primers. The appearance of PCR product of approximately 1,190 bp fragment indicated the presence of plasmid containing the inserted CCR5 promoter sequence. The results of the PCR confirmation were shown in Figure 11.

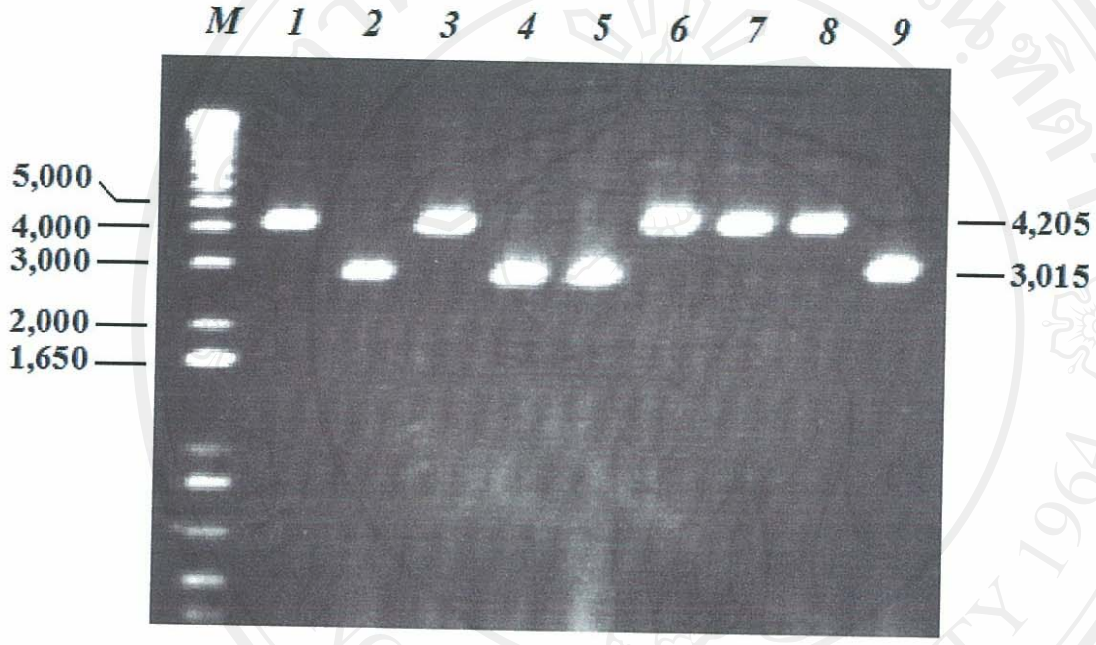

Figure 10 Illustration of DNA extracted from the bacterial colonies after selected by growing on the medium containing 100 µg/ml of ampicillin. A 1 Kb Plus DNA Ladder was used as a marker (Lane ***M***). Lane ***1***, ***3***, ***6-8*** show an approximate 4.2 kb of plasmid DNA of H02 clone number 1, 3, 6-8, respectively. Lane ***2***, ***4-5***, ***9*** show an approximately 3.0 kb empty plasmid of H02 clone number 2, 4-5, and 9, respectively.

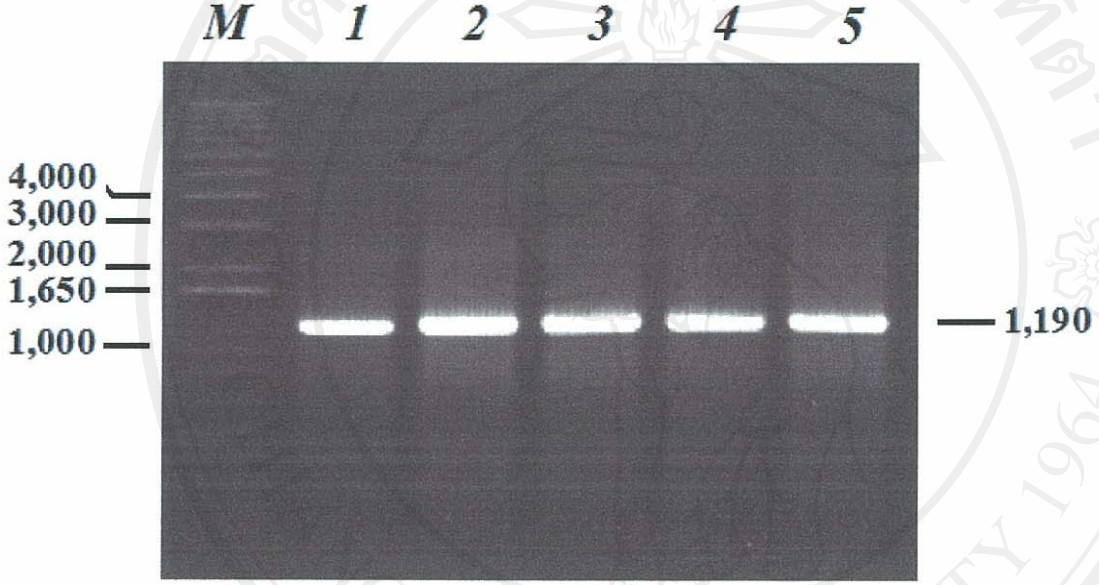

Figure 11 PCR amplification product of CCR5 promoter region from the 4.2 kb of the recombinant clones using R5-PF and R5-PR primers. Lane ***M*** shows 1 Kb Plus DNA Ladder marker. Lane ***1-5*** show the PCR product from H02 clone number 1, 3, 6-8, respectively.

2.3 The polymorphism of CCR5 promoter region

The nucleotide sequence of the CCR5 promoter region was determined by dye terminator cycle sequencing method using the Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing Kit (Amersham Bioscience, England). The CCR5 promoter region was sequenced in both directions by using modified M13 universal primer and R5-PR primer. The Modified M13 Universal primer which specified to the nucleotide sequence on the pGEM®-T Easy Vector was used to sequence in the forward direction, while the R5-PR primer was used to sequence in the backward direction. The Long-Read Tower System version 3.1 (Visible Genetic, USA) was used to resolve this sequence data. Examples of electrophoregram of the nucleotide sequences of the CCR5 promoter region from HEPS individual (H07) on forward and backward direction are shown in Figure 12 and 13, respectively.

In order to analyze the polymorphisms, the nucleotide sequences of the CCR5 promoter region obtained from HEPS and their HIV-1 seropositive spouses were compared with the CCR5 sequence at position 58,548 to 59,737 that reported in the GenBank (accession number U95626). The CCR5 promoter sequences obtained from HEPS individuals and their HIV-1 seropositive spouses are shown in Figure 14 and 15, respectively.

Figure 12 The electrophoregram of the nucleotide sequence in the CCR5 promoter region of the HEPS (H07) on forward direction using Modified M13 Universal primer.

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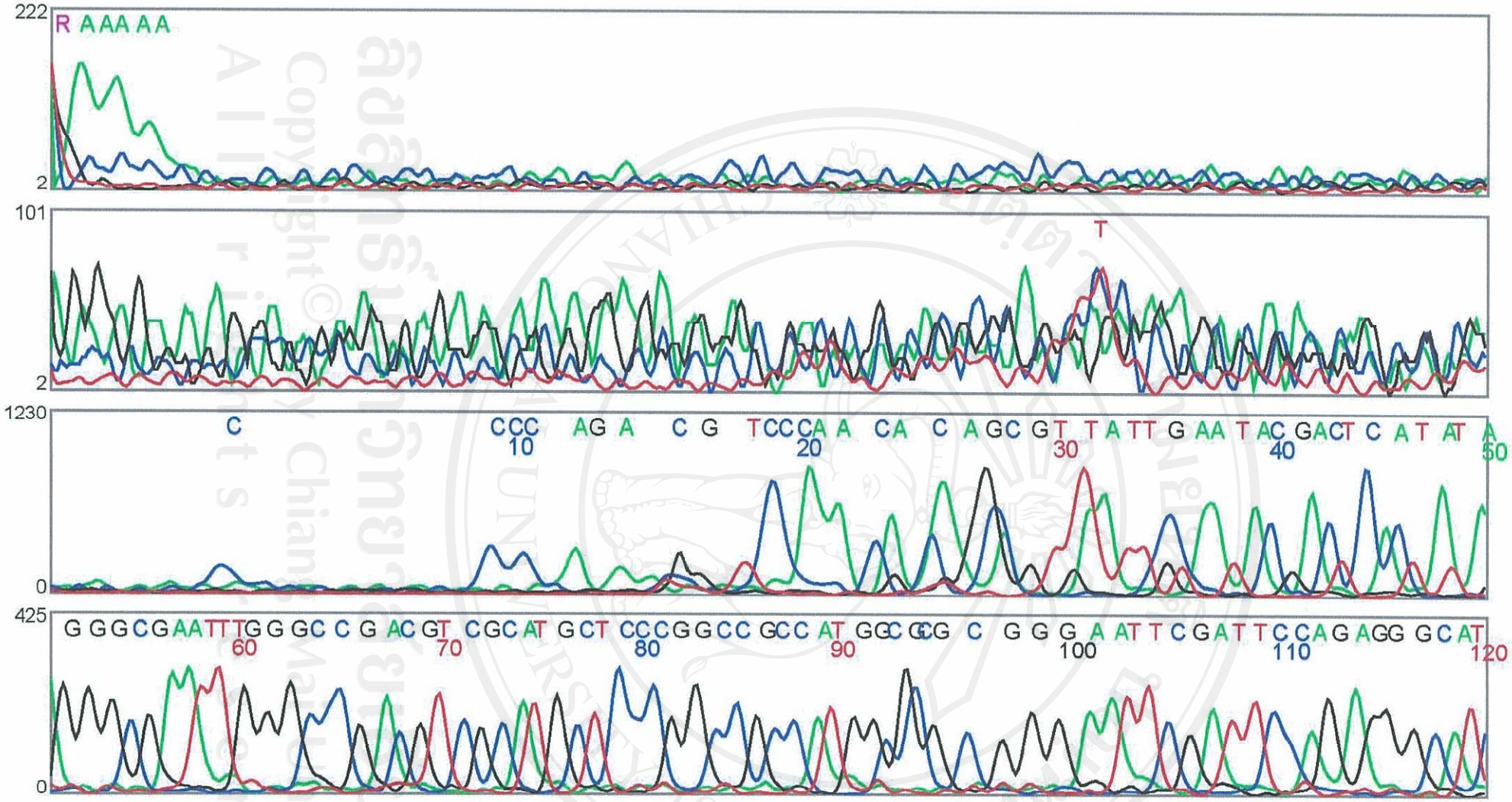

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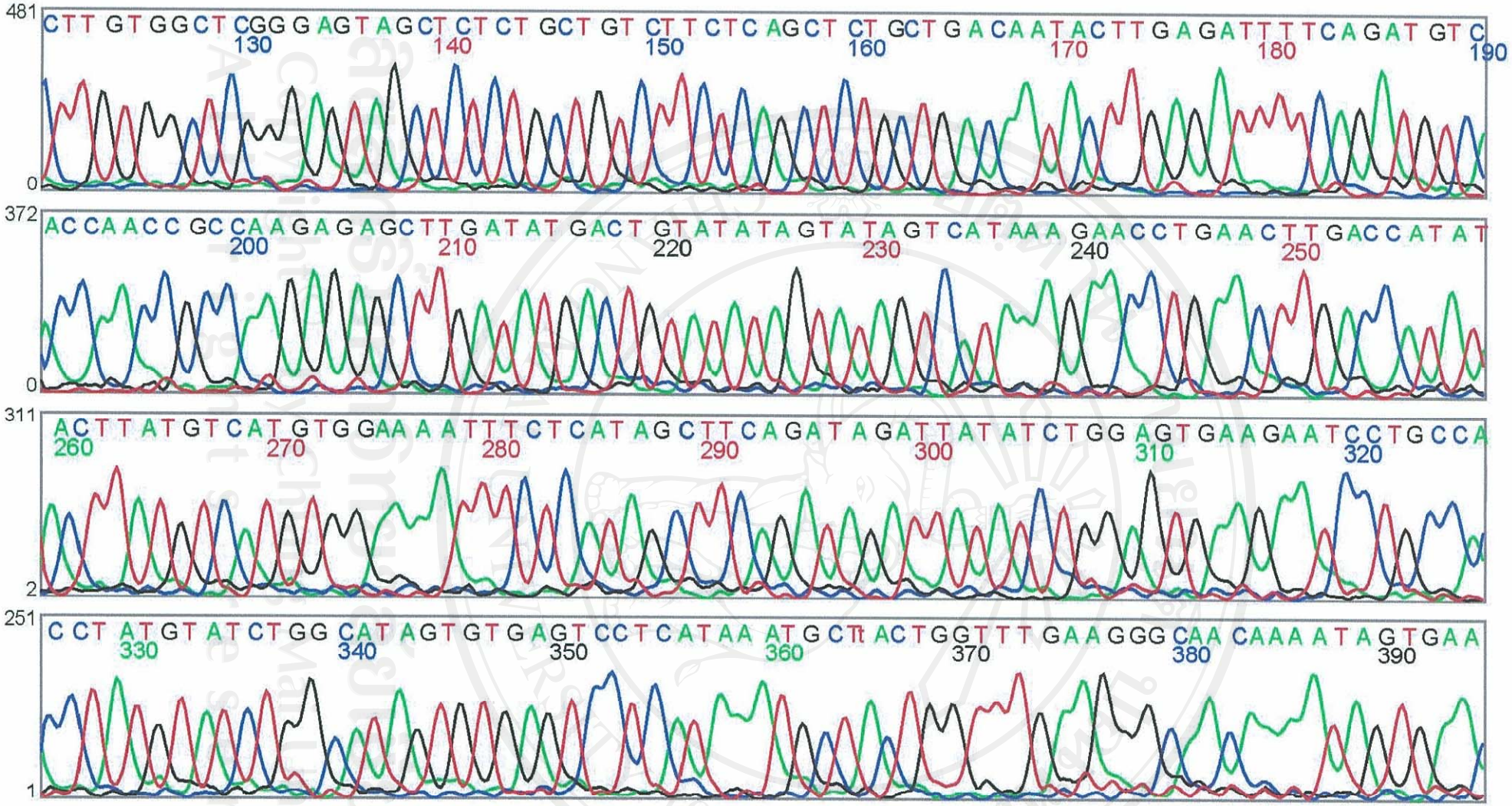

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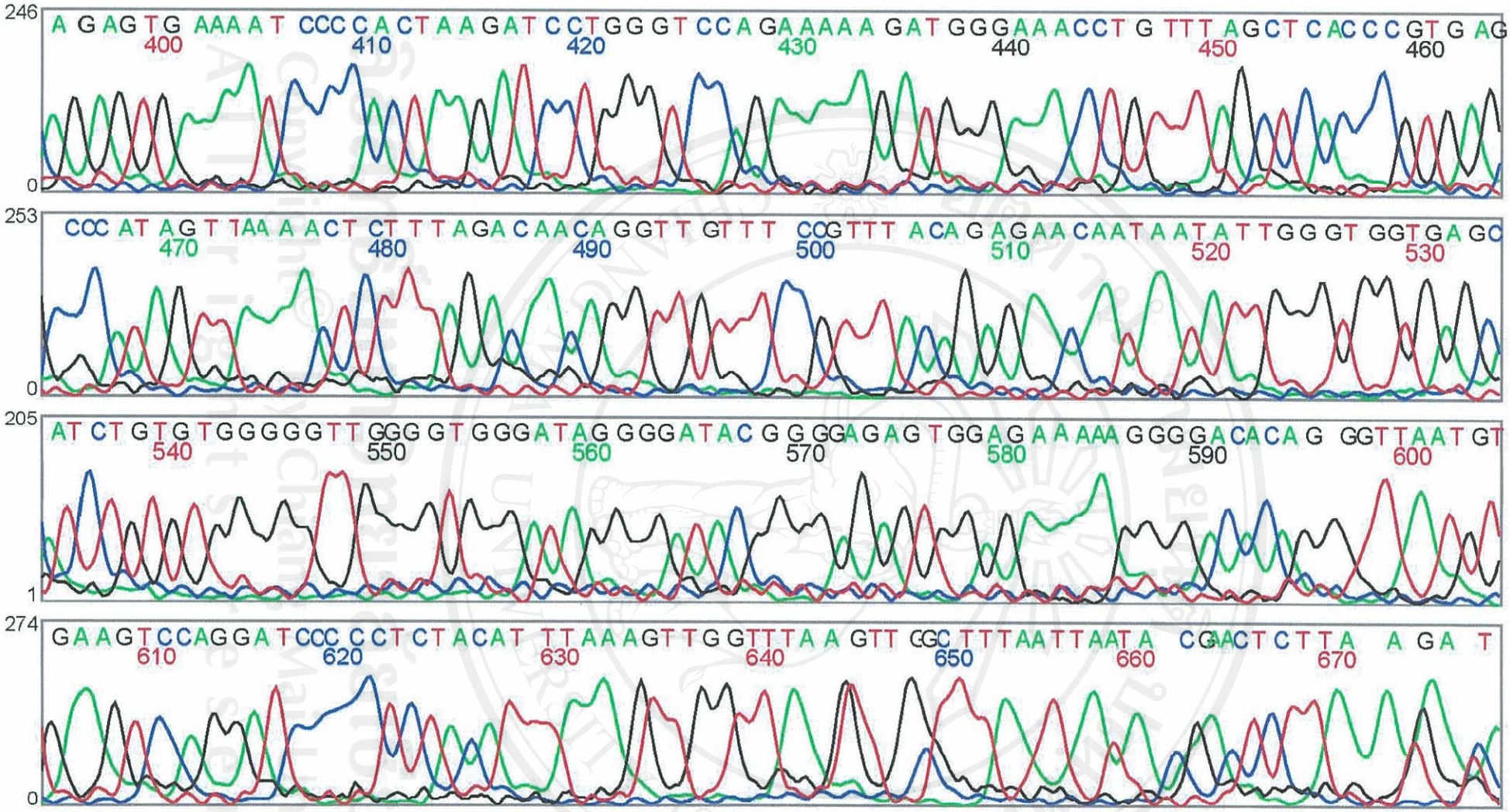

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Figure 13 The electrophoregram of the nucleotide sequence in the CCR5 promoter region of the HEPS (H07) on backward direction using R5-PR primer.

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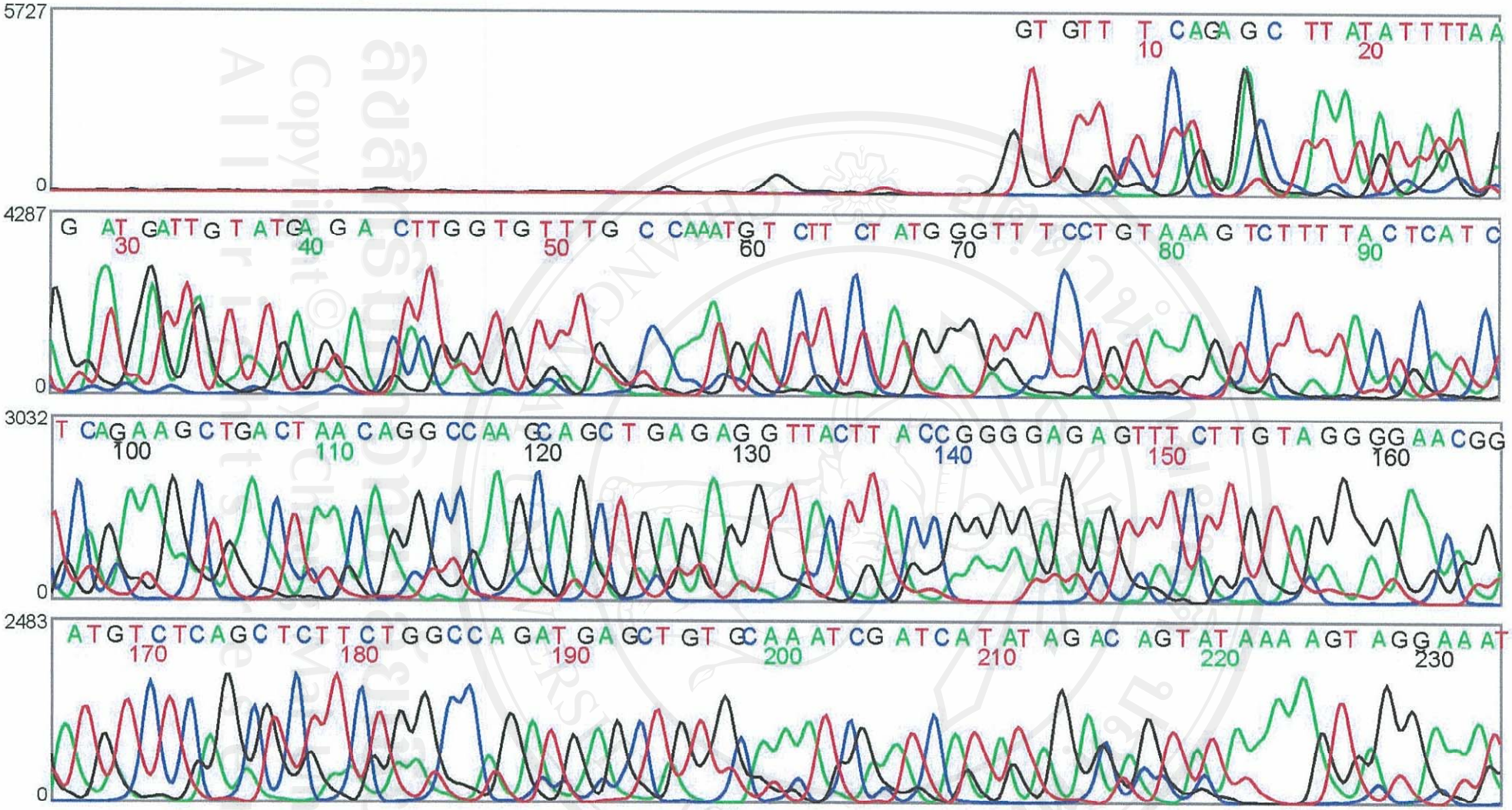

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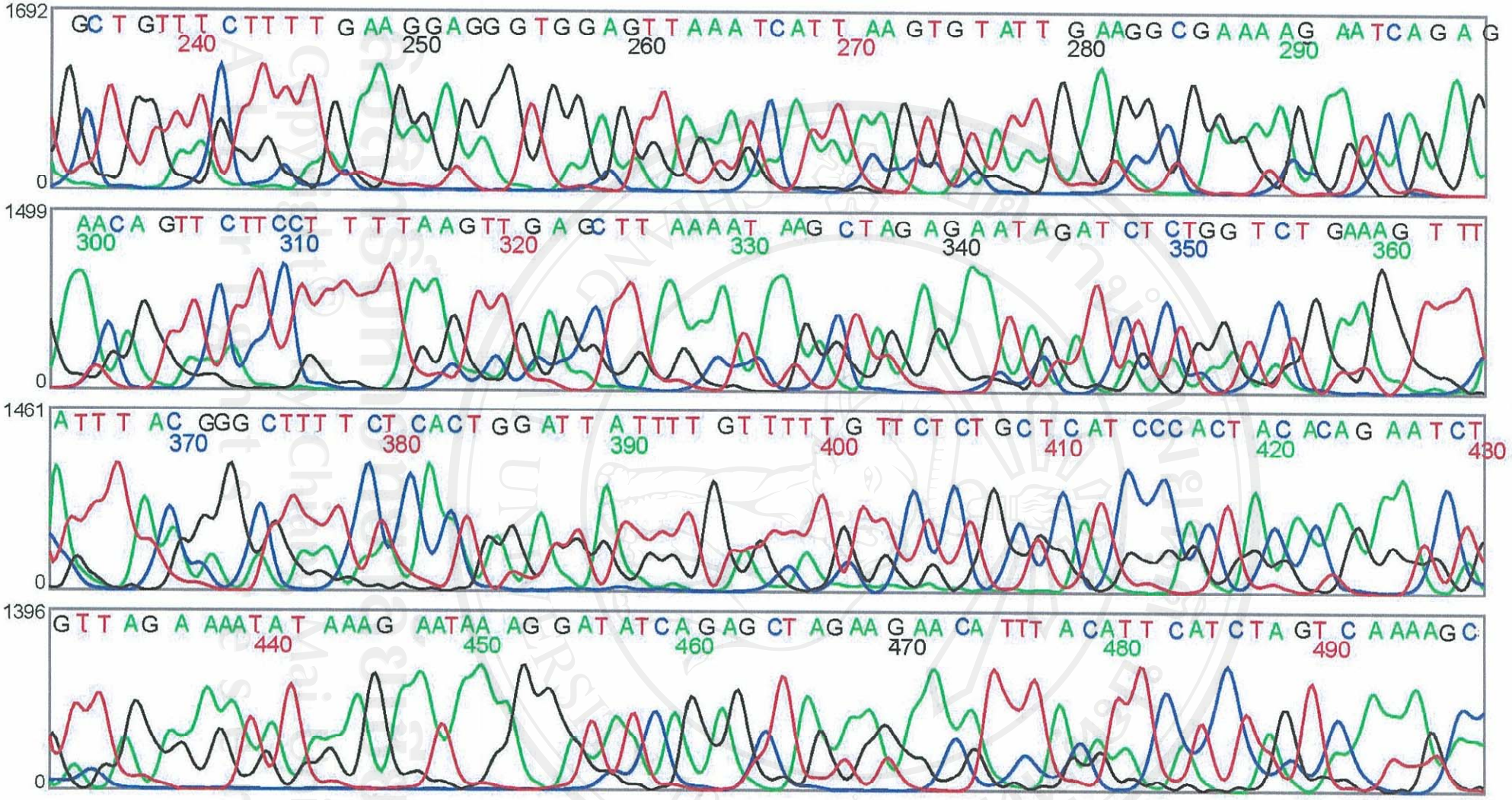

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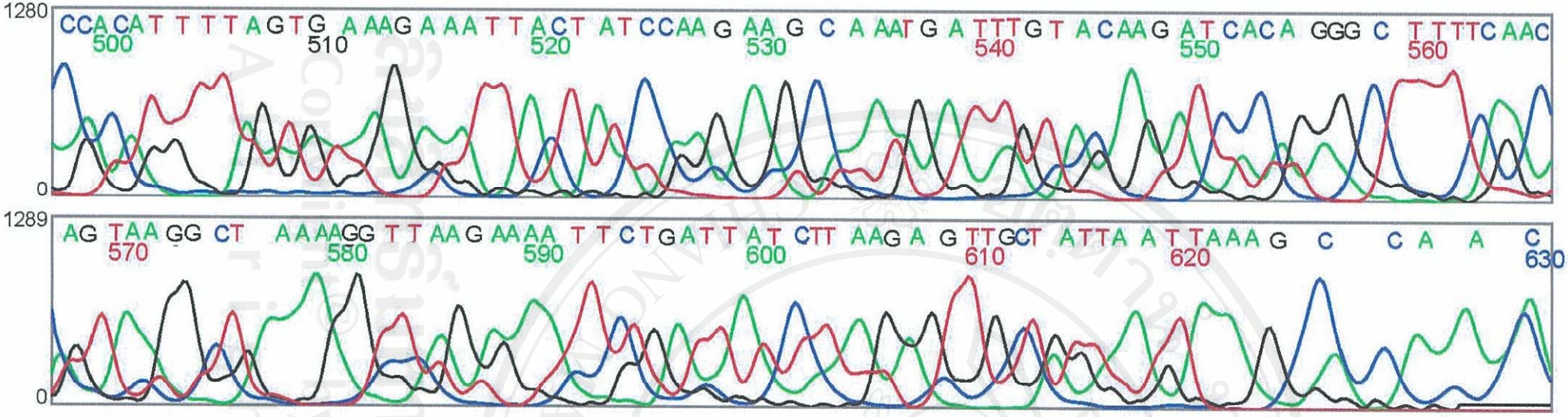

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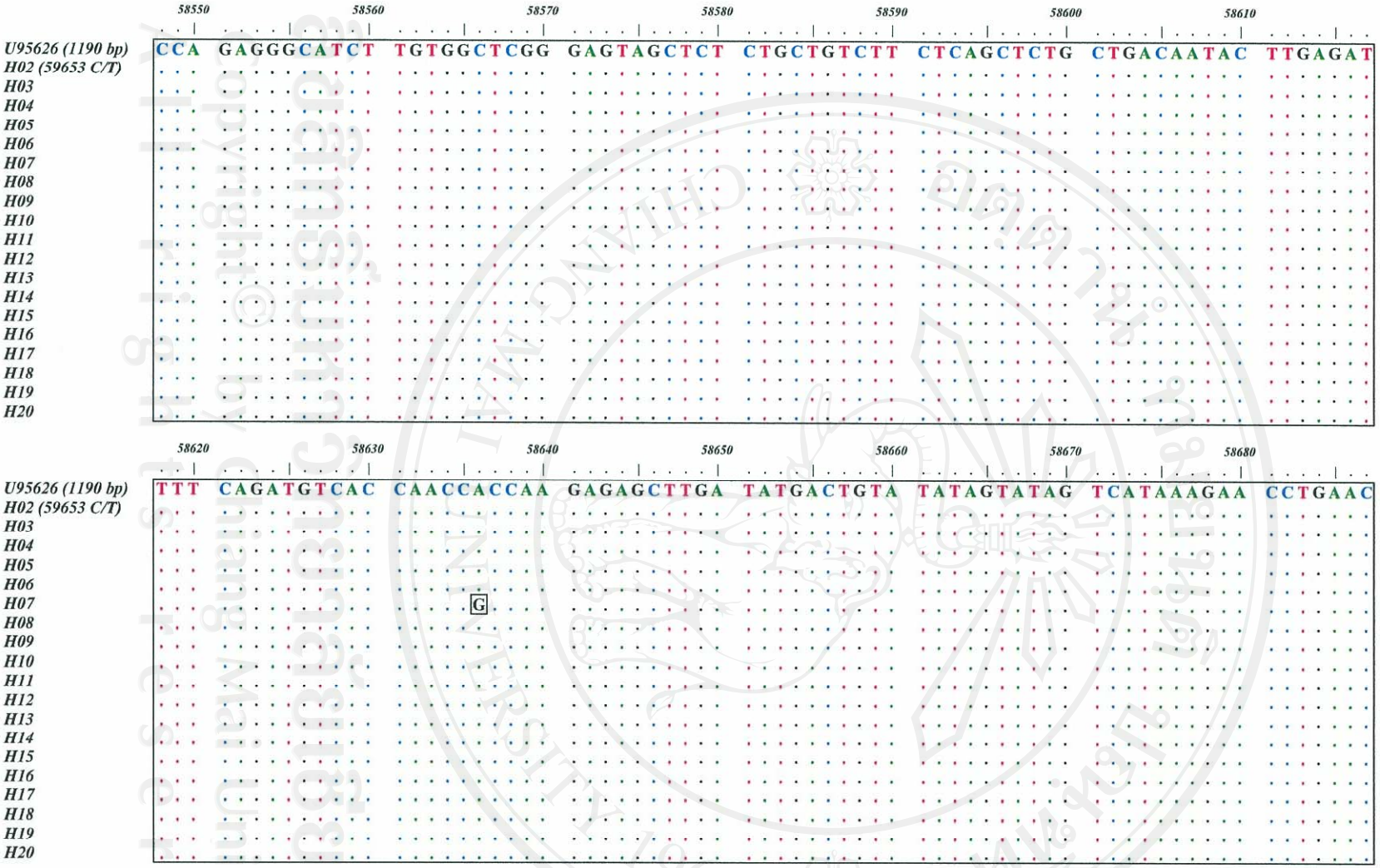
Figure 14 Comparison of the CCR5 promoter sequences obtained from the HEPS subjects with the corresponding position 58,548 to 59,737 of CCR5 sequences accession number U95626 submitted in the GenBank database.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58610
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H02 (59653 C/T)
H03
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58620
58630
58640
58650
58660
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U95626 (1190 bp) TTT CAGATGTCAC CAACCACCAA GAGAGCTTGA TATGACTGTA TATAGTATAG TCATAAAGAA CCTGAAC
H02 (59653 C/T)
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H07 G
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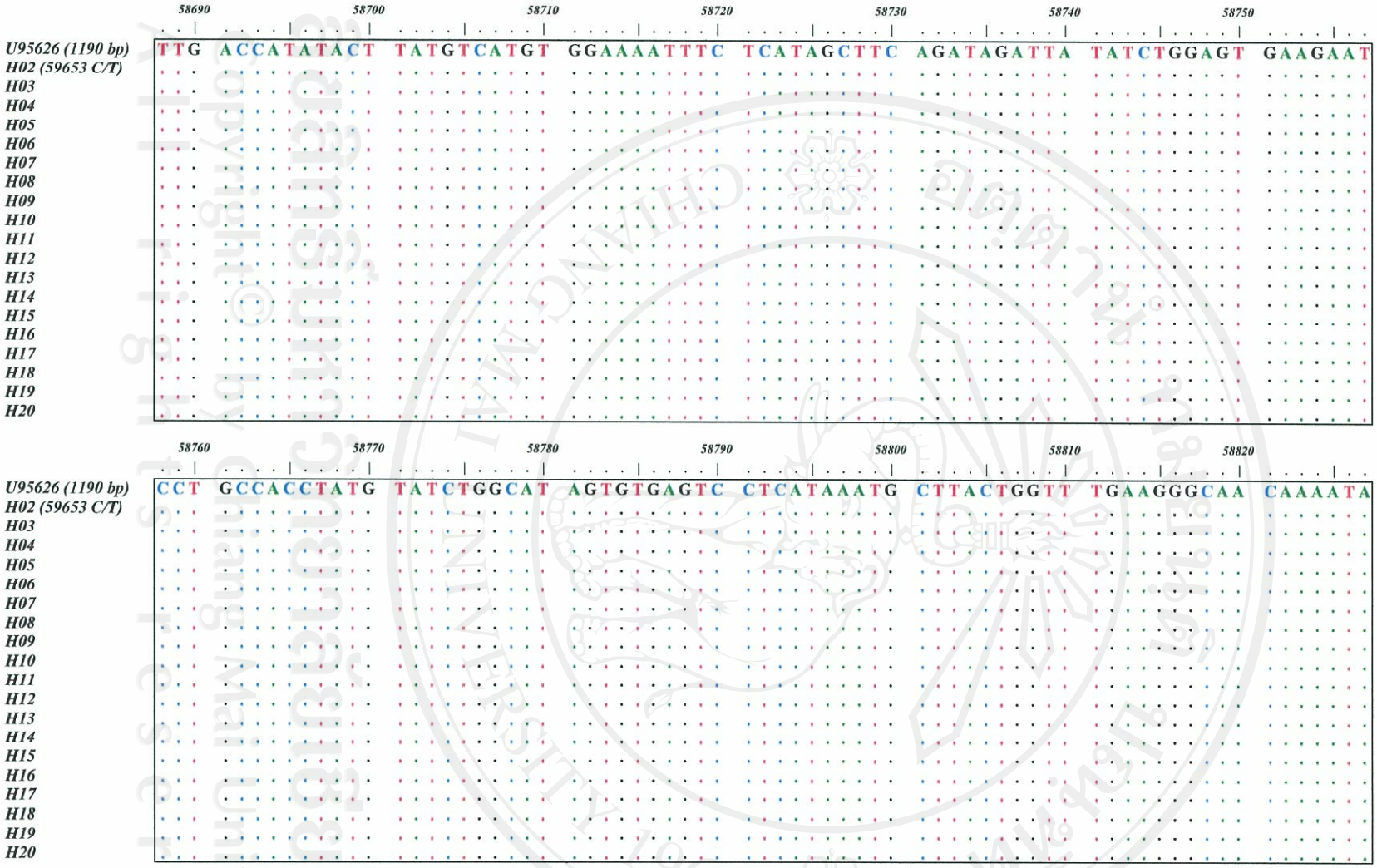
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H02 (59653 C/T)
H03
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58760
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58820
U95626 (1190 bp) CCT GCCACCTATG TATCTGGCAT AGTGTGAGTC CTCATAAATG CTTACTGGTT TGAAGGGCAA CAAAATA
H02 (59653 C/T)
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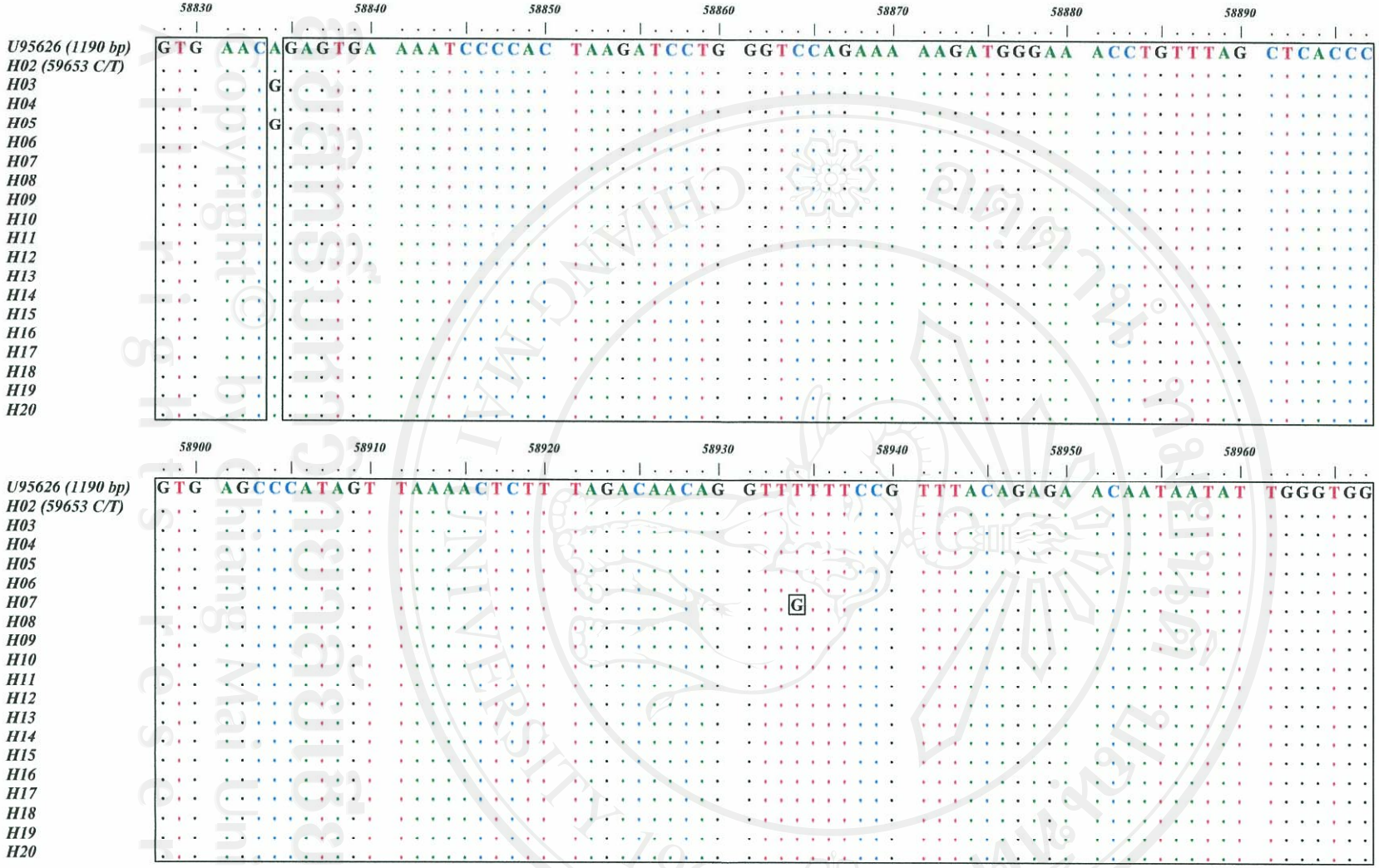
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58870
58880
58890
U95626 (1190 bp) GTG AACAGAGTGA AAATCCCCAC TAAGATCCTG GGTCCAGAAA AAGATGGGAA ACCTGTTTAG CTCACCC
H02 (59653 C/T)
H03 G
H04
H05 G
H06
H07
H08
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58900
58910
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58930
58940
58950
58960
U95626 (1190 bp) GTG AGCCCATAGT TAAAACTCTT TAGACAACAG GTTTTTTCCG TTTACAGAGA ACAATAATAT TGGGTGG
H02 (59653 C/T)
H03
H04
H05
H06
H07 G
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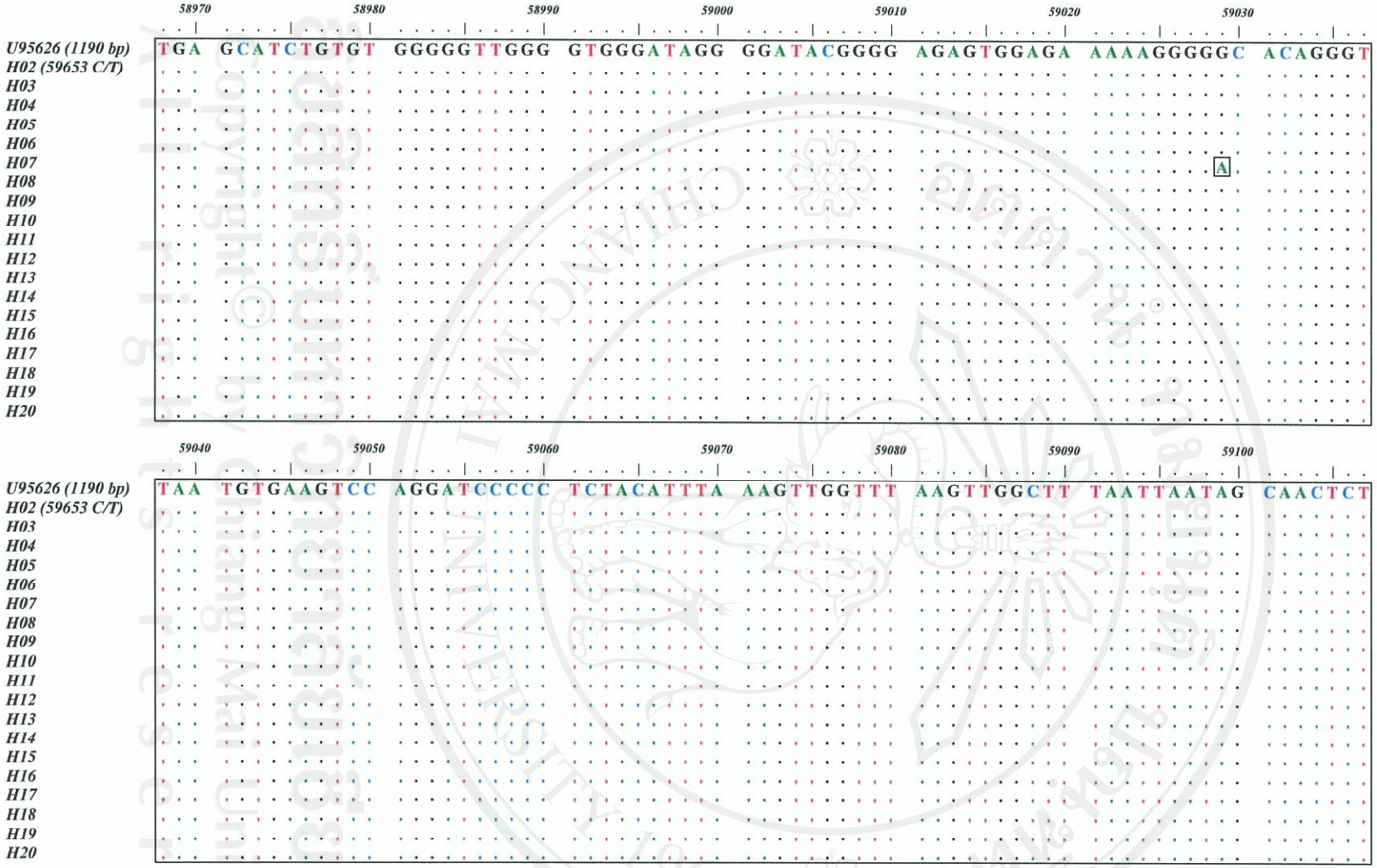
H20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H02 (59653 C/T)
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59040
59050
59060
59070
59080
59090
59100
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H02 (59653 C/T)
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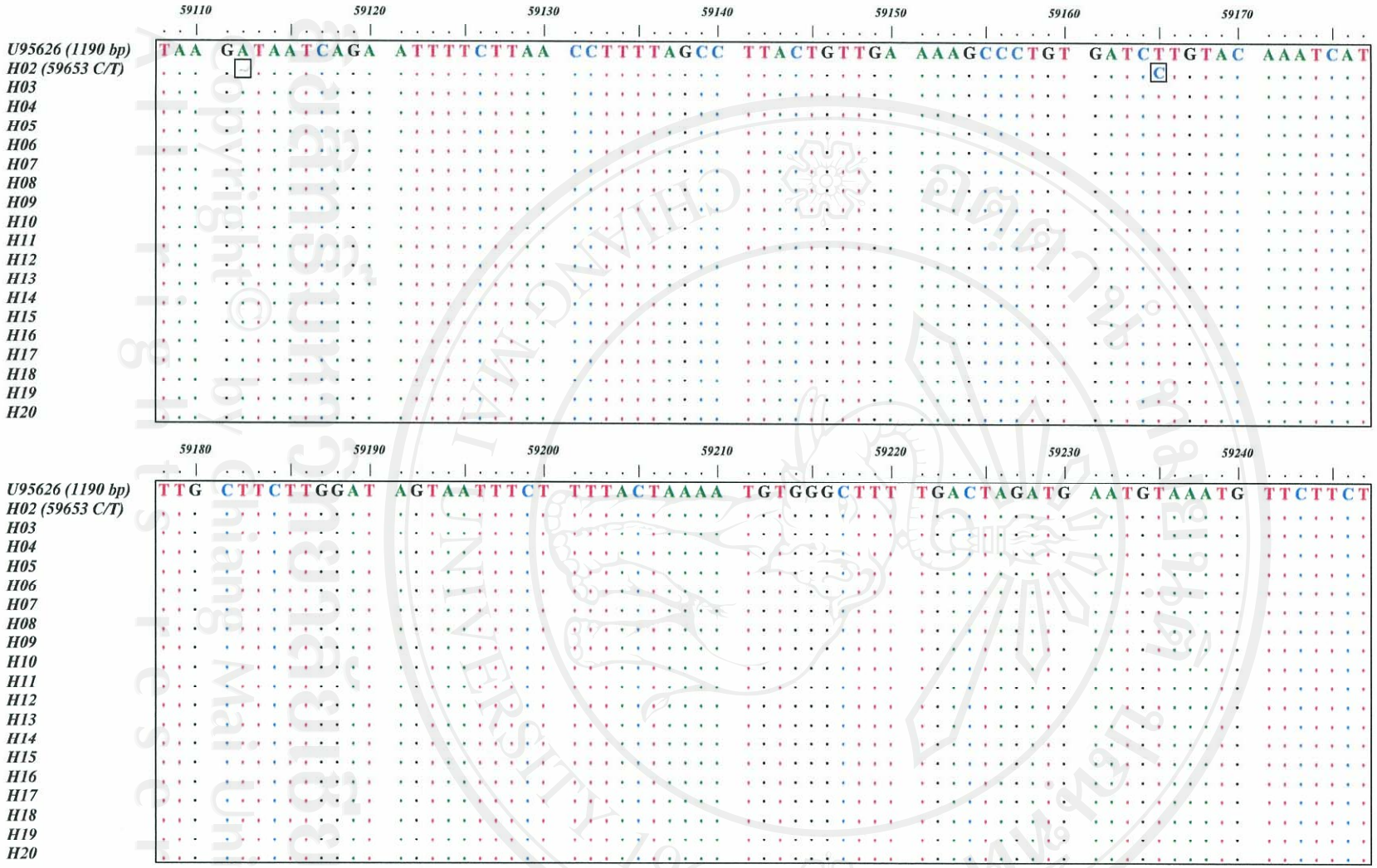
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H02 (59653 C/T)
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59180
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59200
59210
59220
59230
59240
U95626 (1190 bp) TTG CTTCTTGGAT AGTAATTTCT TTTACTAAAA TGTGGGCTTT TGACTAGATG AATGTAAATG TTCTTCT
H02 (59653 C/T)
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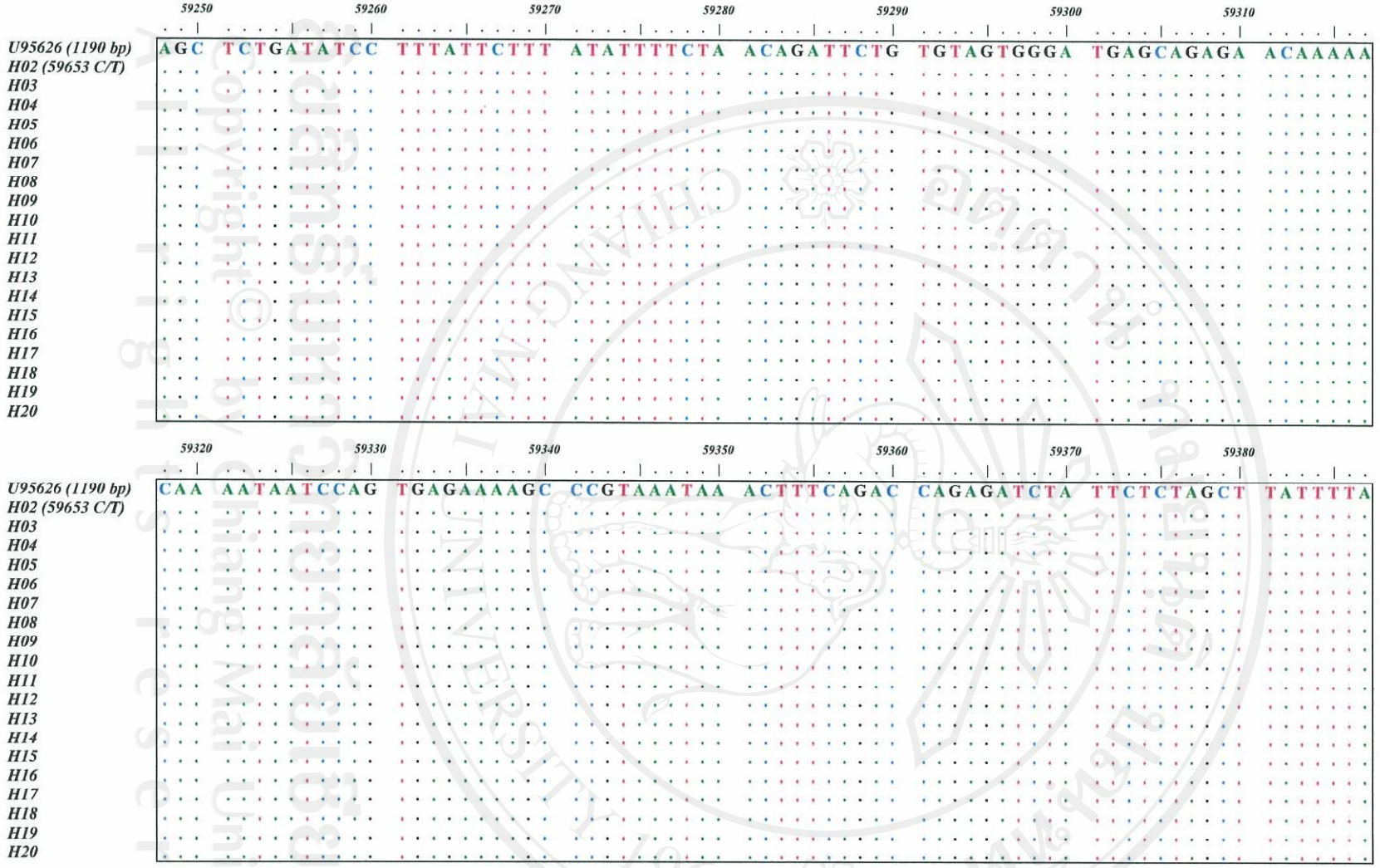
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H02 (59653 C/T)
H03
H04
H05
H06
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H14
H15
H16
H17
H18
H19
H20
59320
59330
59340
59350
59360
59370
59380
U95626 (1190 bp) CAA AATAATCCAG TGAGAAAAGC CCGTAAATAA ACTTTCAGAC CAGAGATCTA TTCTCTAGCT TATTTTA
H02 (59653 C/T)
H03
H04
H05
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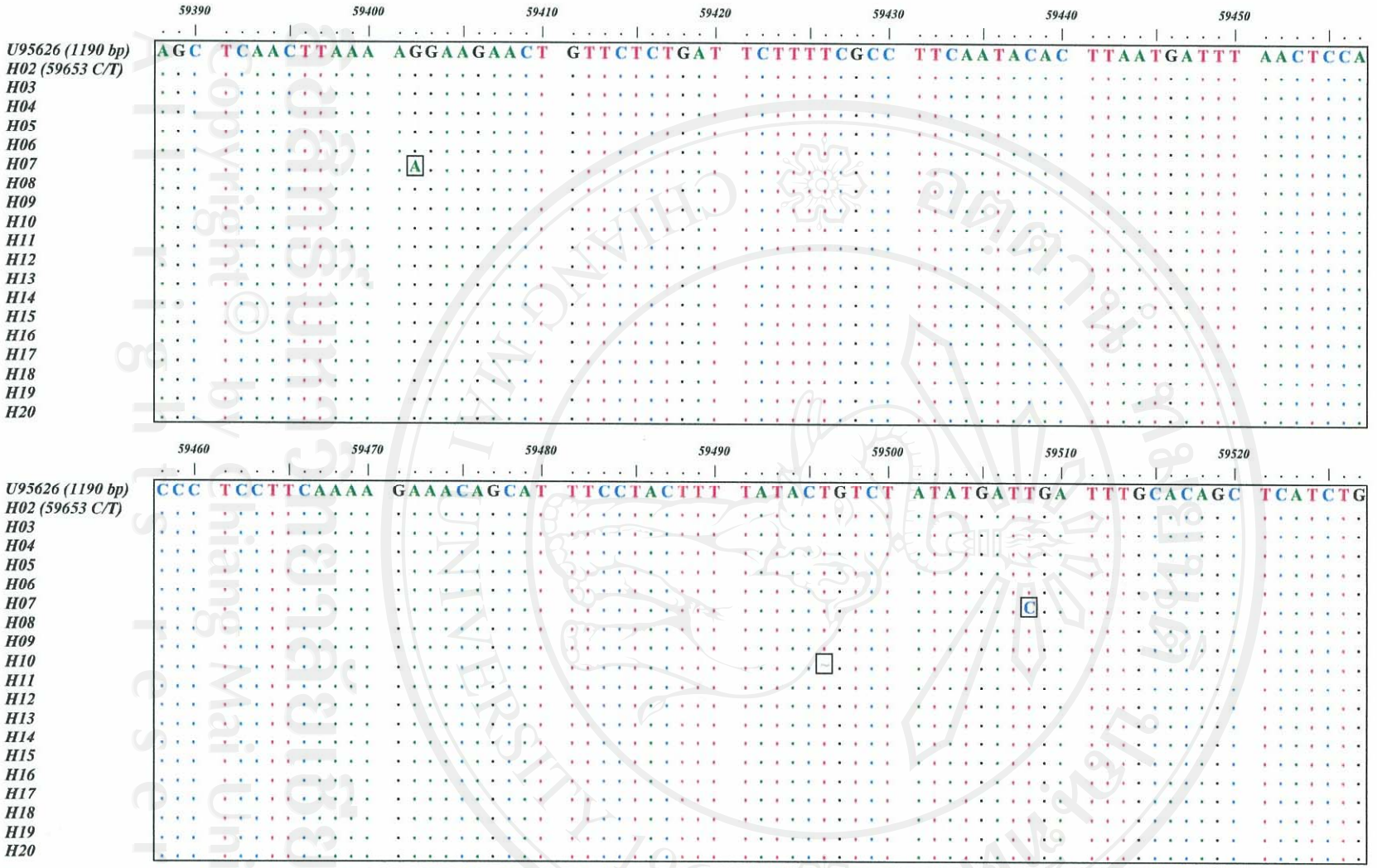
H20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H02 (59653 C/T)
H03
H04
H05
H06
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H13
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H15
H16
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H18
H19
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59490
59500
59510
59520
U95626 (1190 bp) CCC TCCTTCAAAA GAAACAGCAT TTCCTACTTT TATACTGTCT ATATGATTGA TTTGCACAGC TCATCTG
H02 (59653 C/T)
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H05
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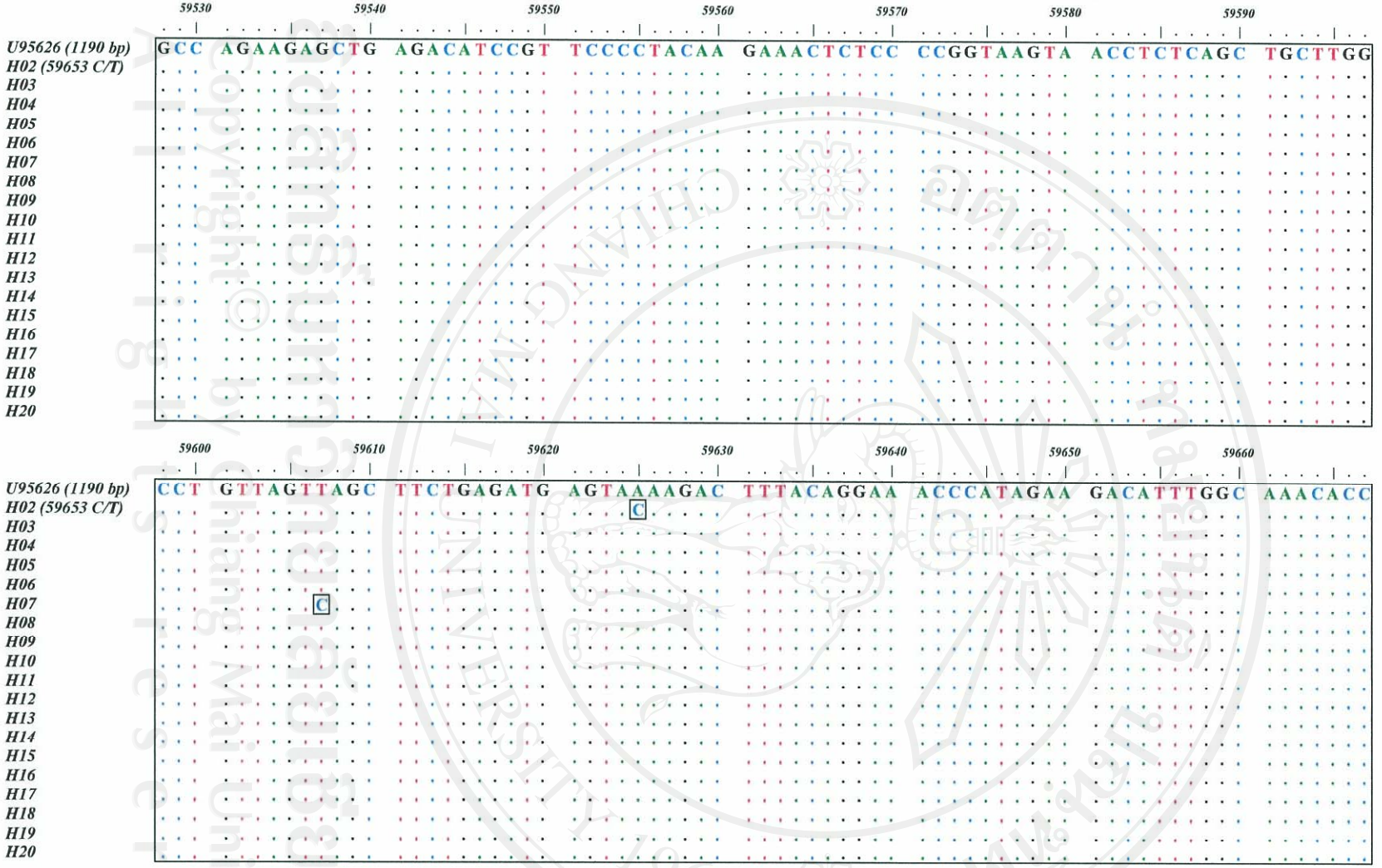
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H02 (59653 C/T)
H03
H04
H05
H06
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H14
H15
H16
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H19
H20
59600
59610
59620
59630
59640
59650
59660
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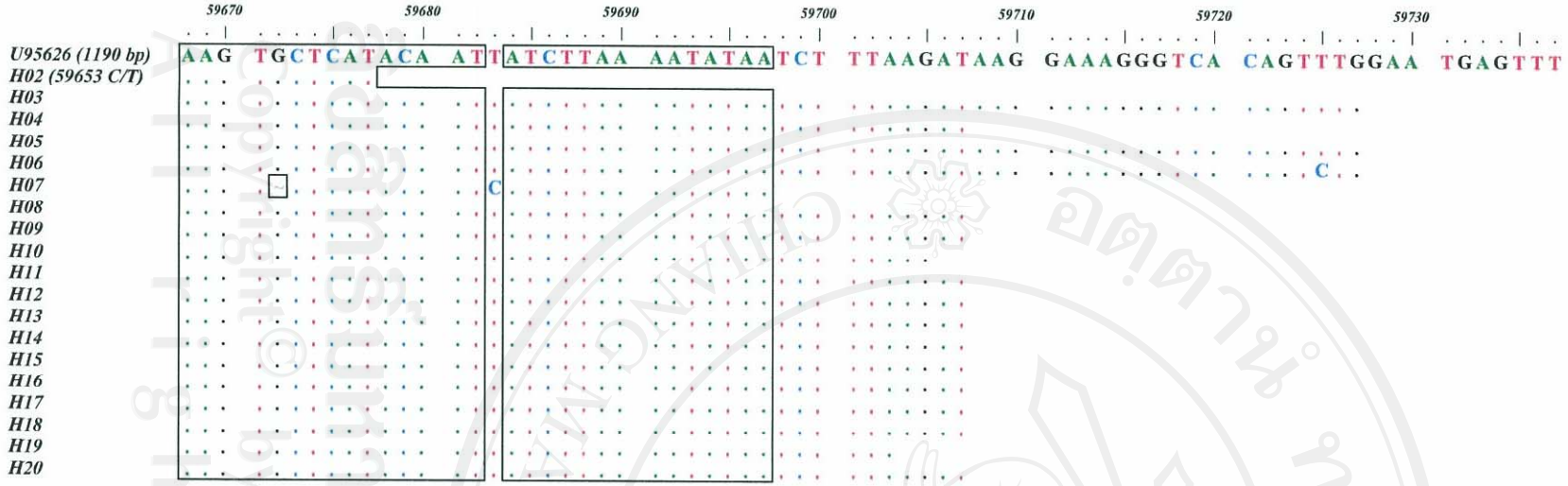
H07 C



U95626 (1190 bp)
H02 (59653 C/T)
H03
H04
H05
H06
H07
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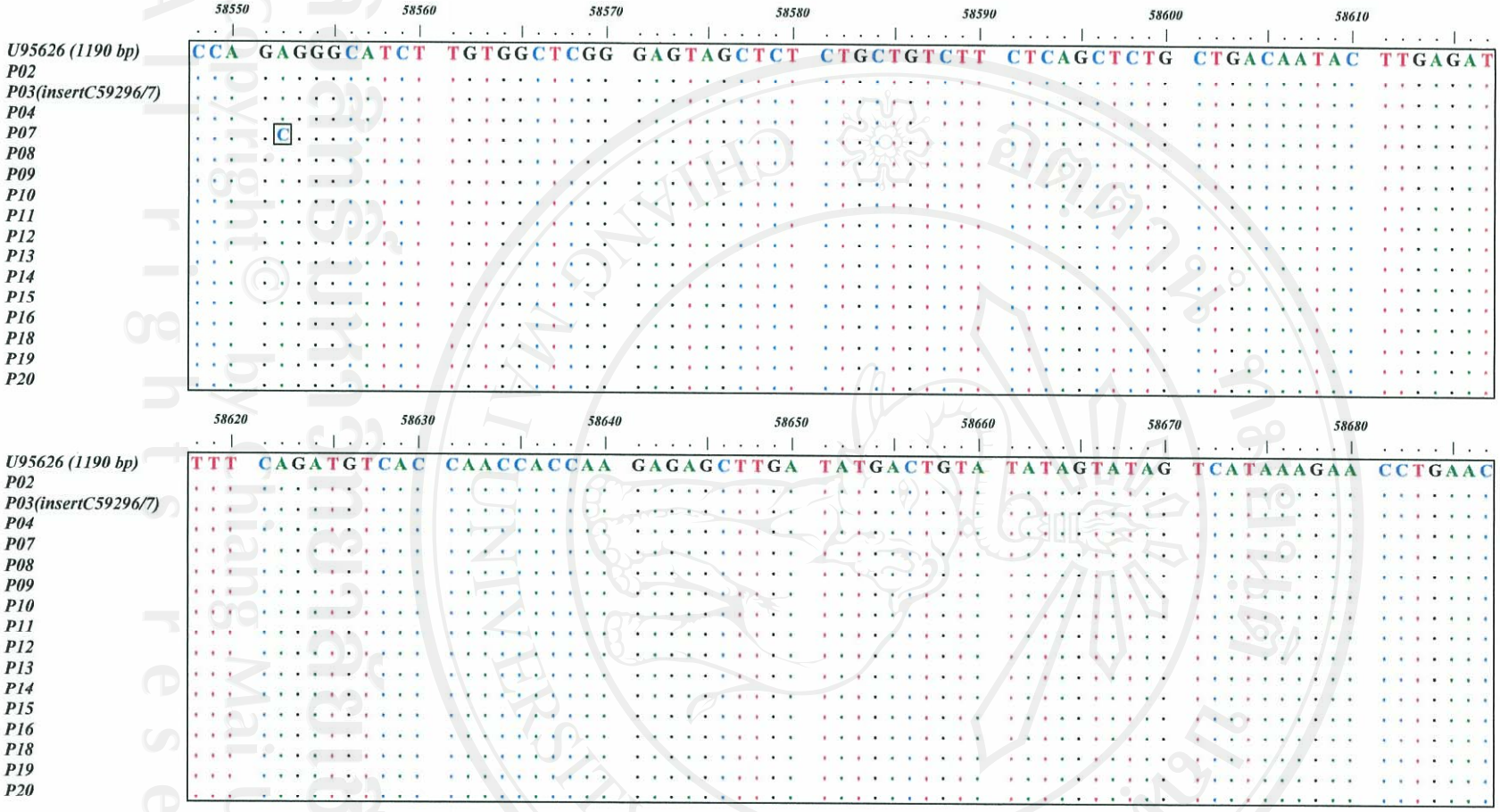
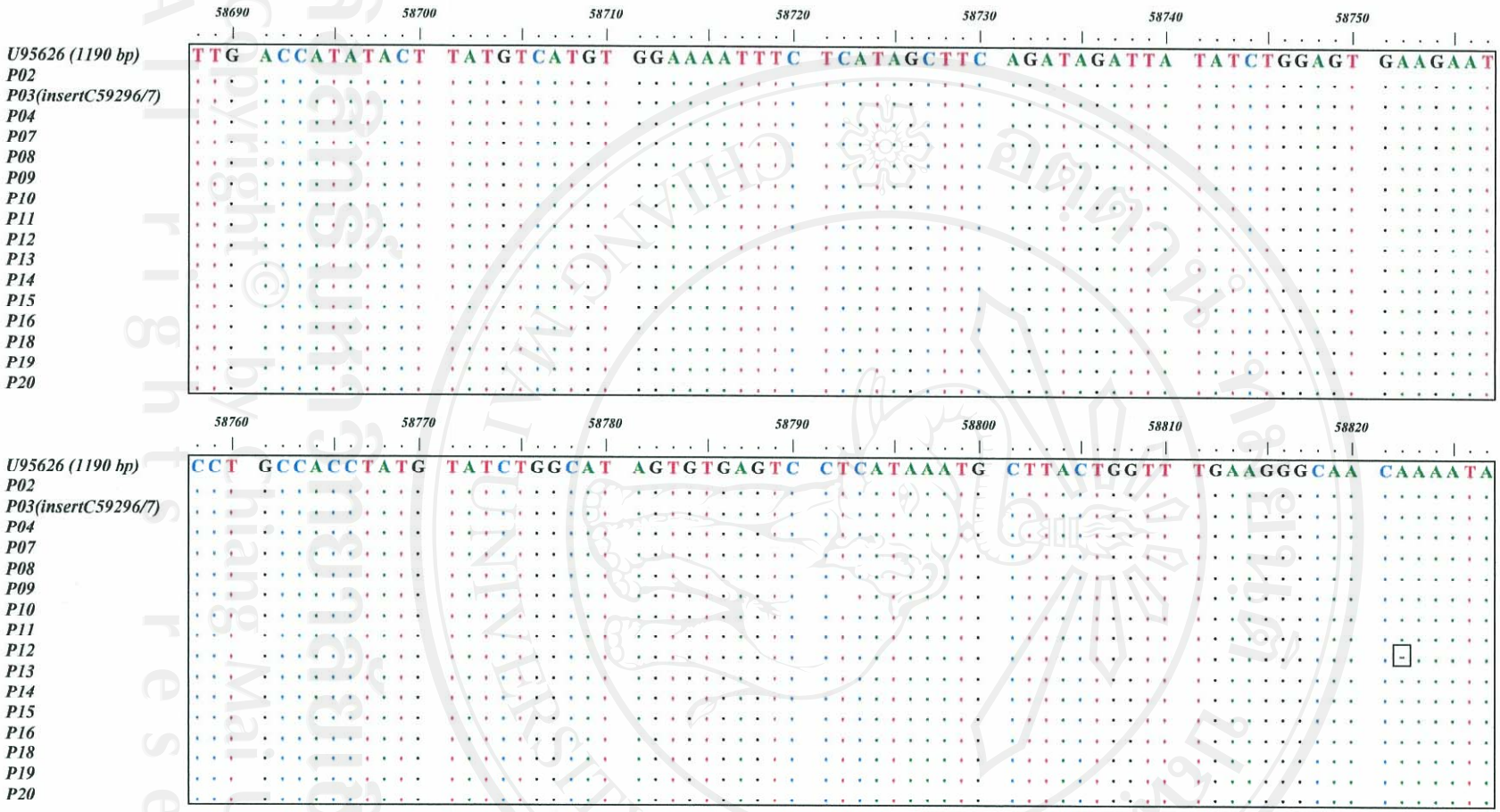
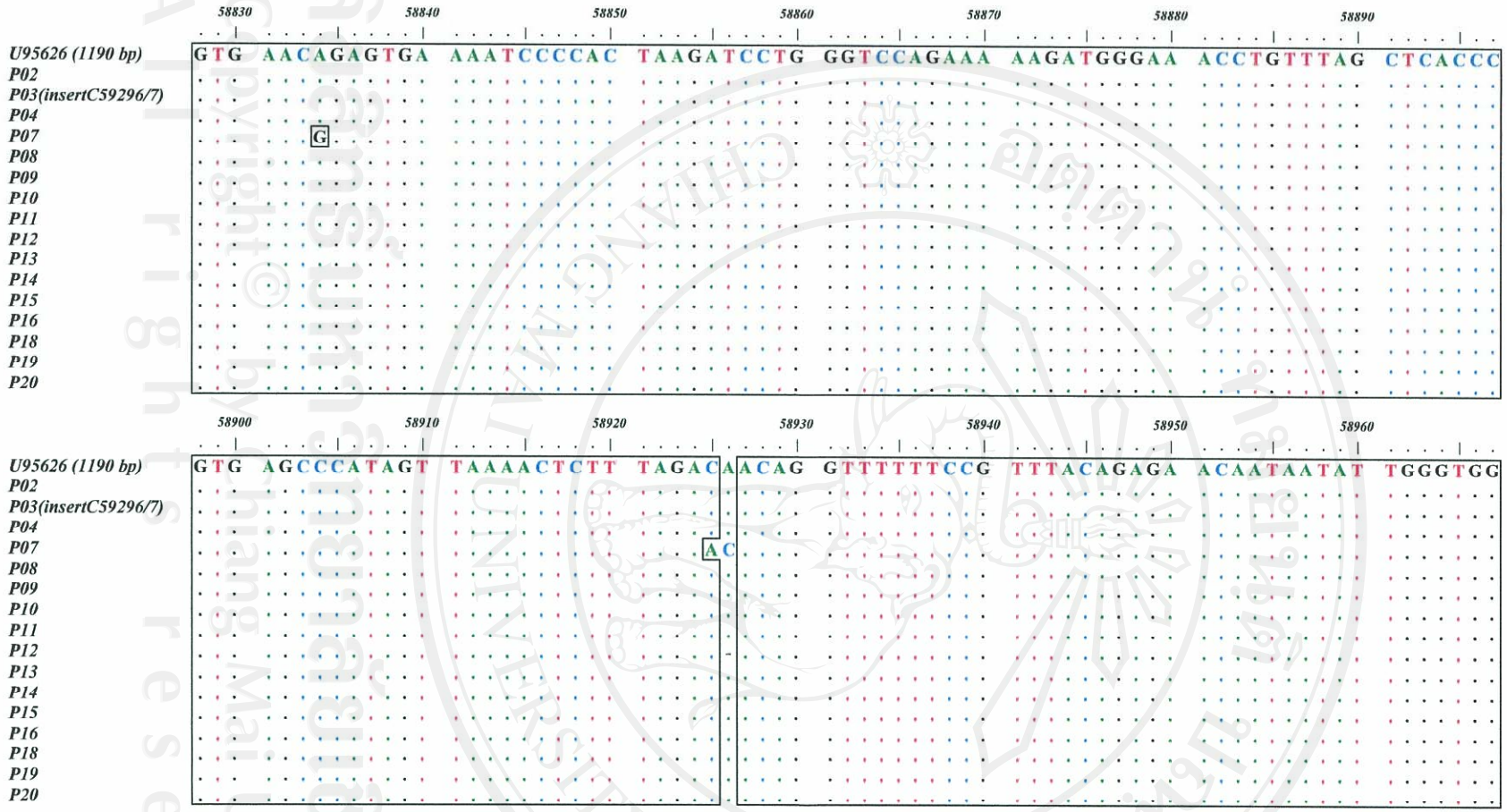
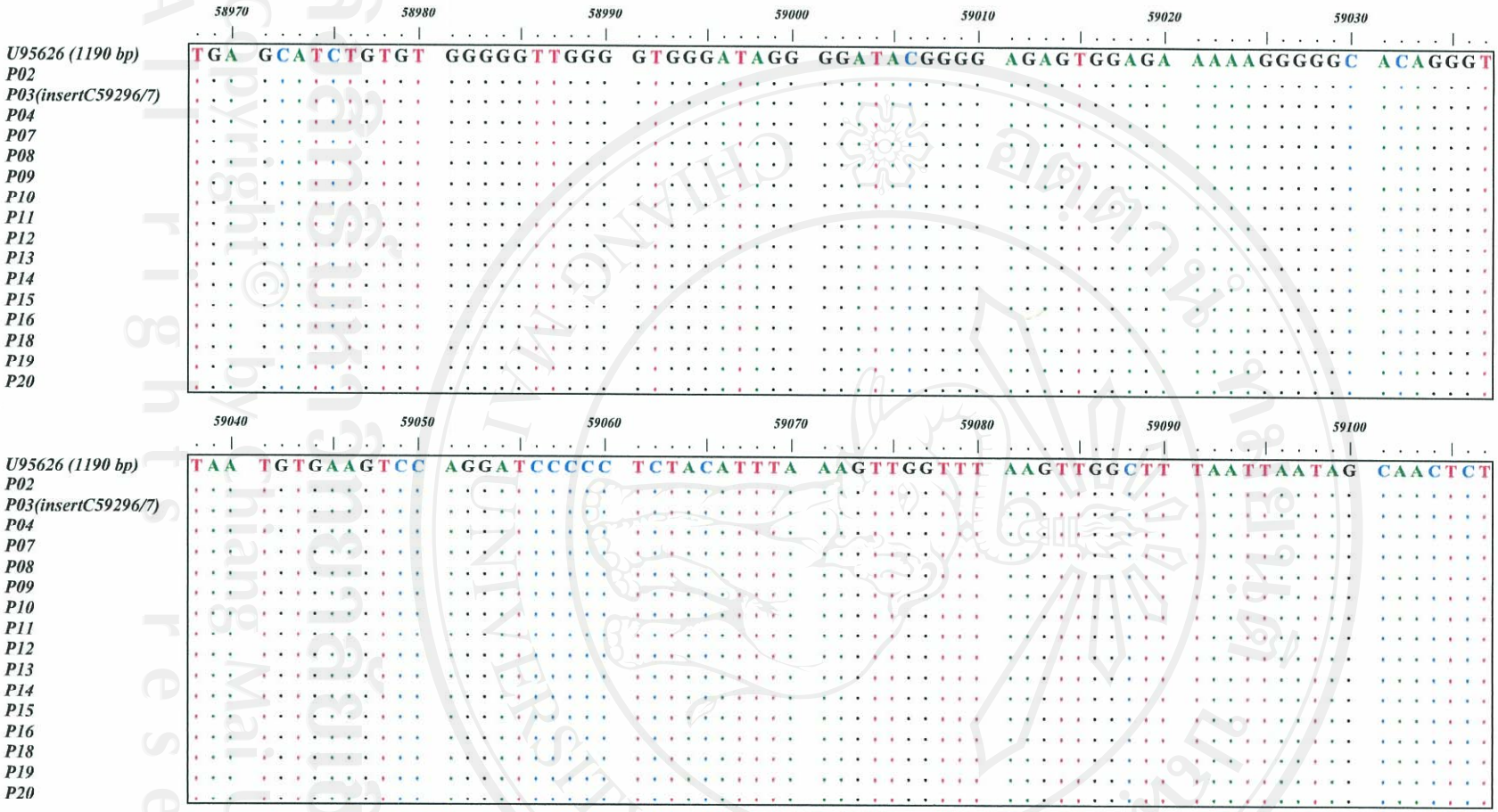
H14
H15
H16
H17
H18
H19
H20

Figure 15 Comparison of the CCR5 promoter sequences of 16 HIV-1 seropositive subjects with the corresponding position 58,548 to 59,737 of CCR5 sequences accession number U95626 submitted in the GenBank database.





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58980
58990
59000
59010
59020
59030
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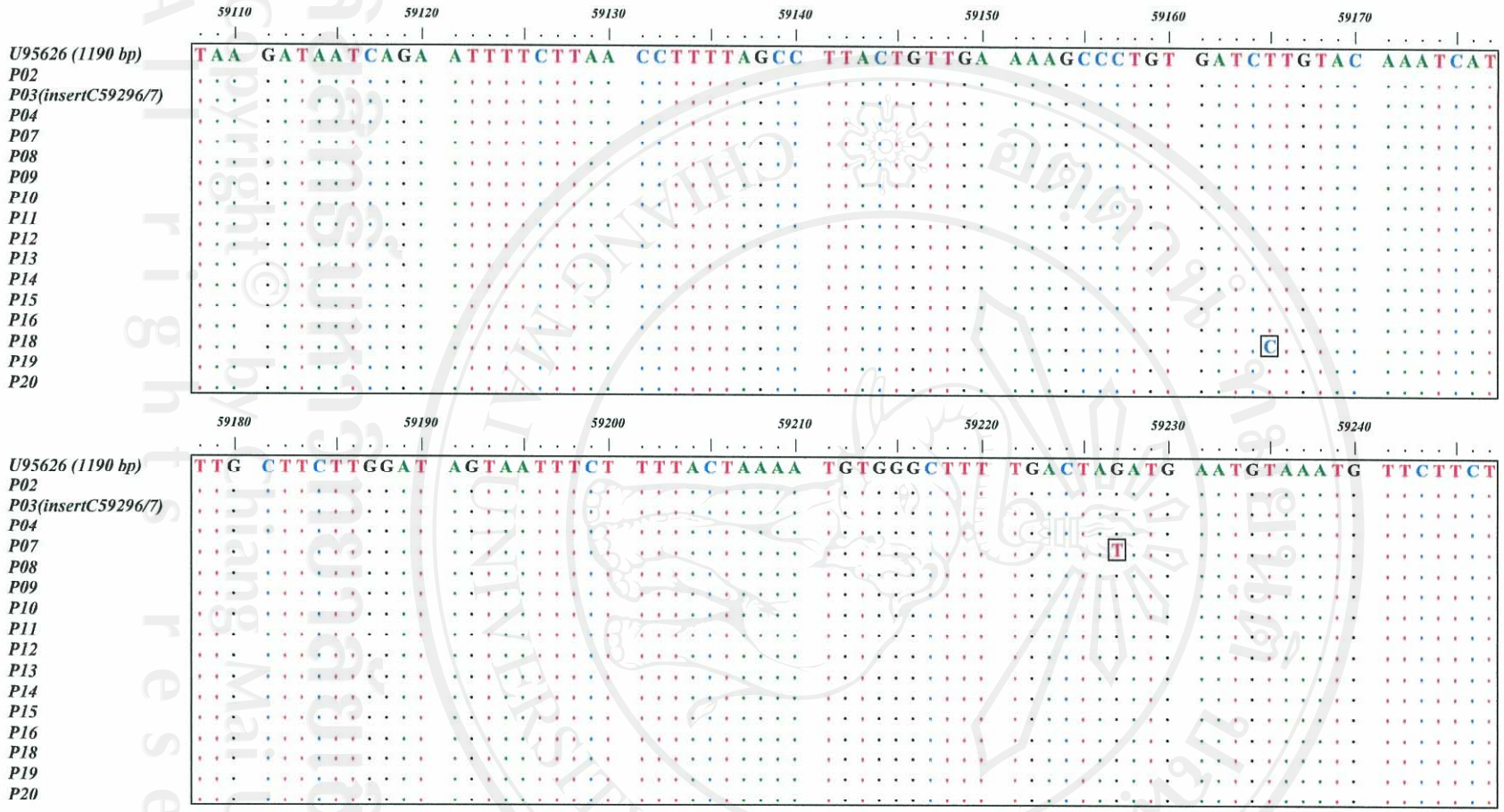
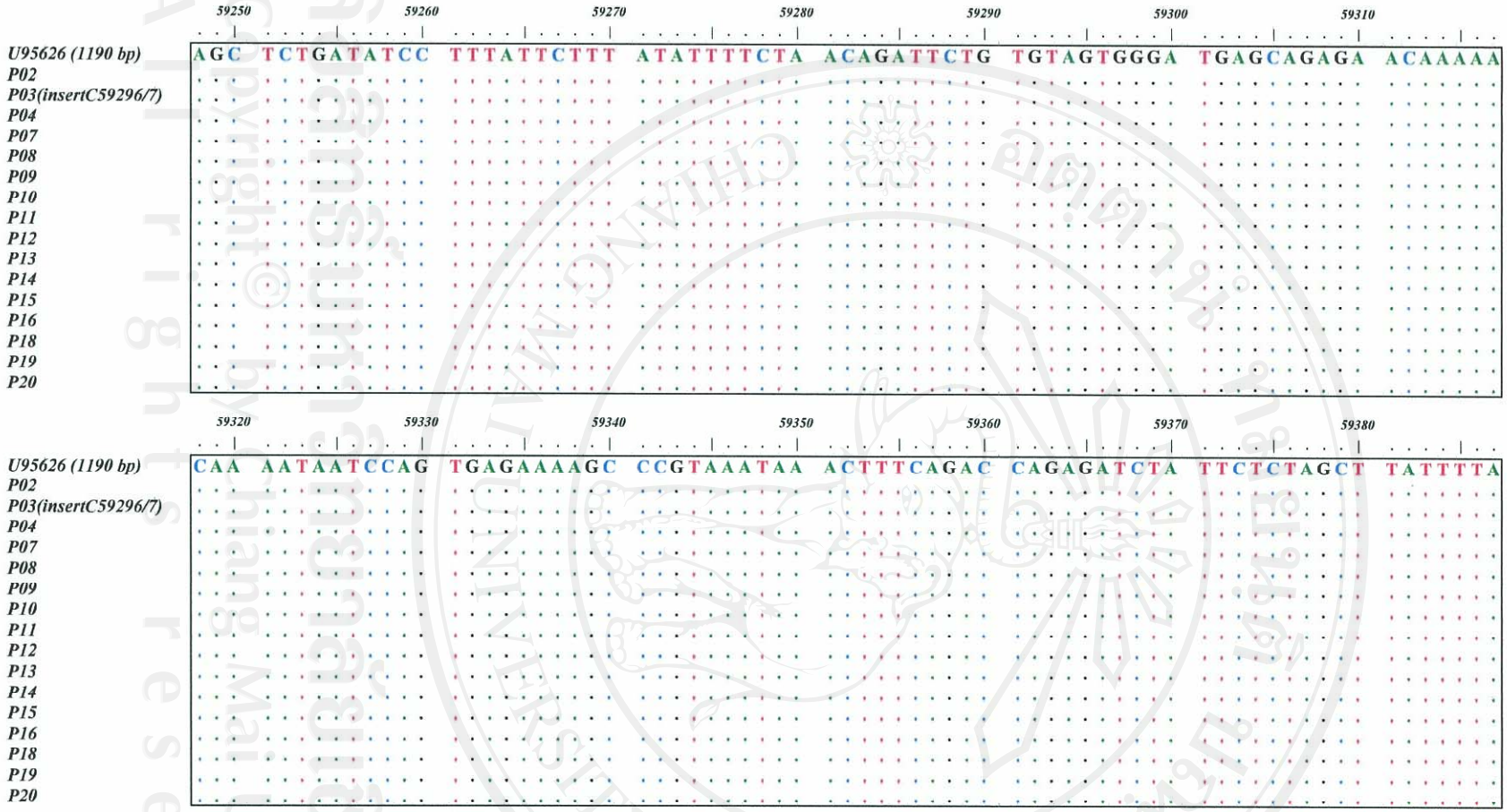
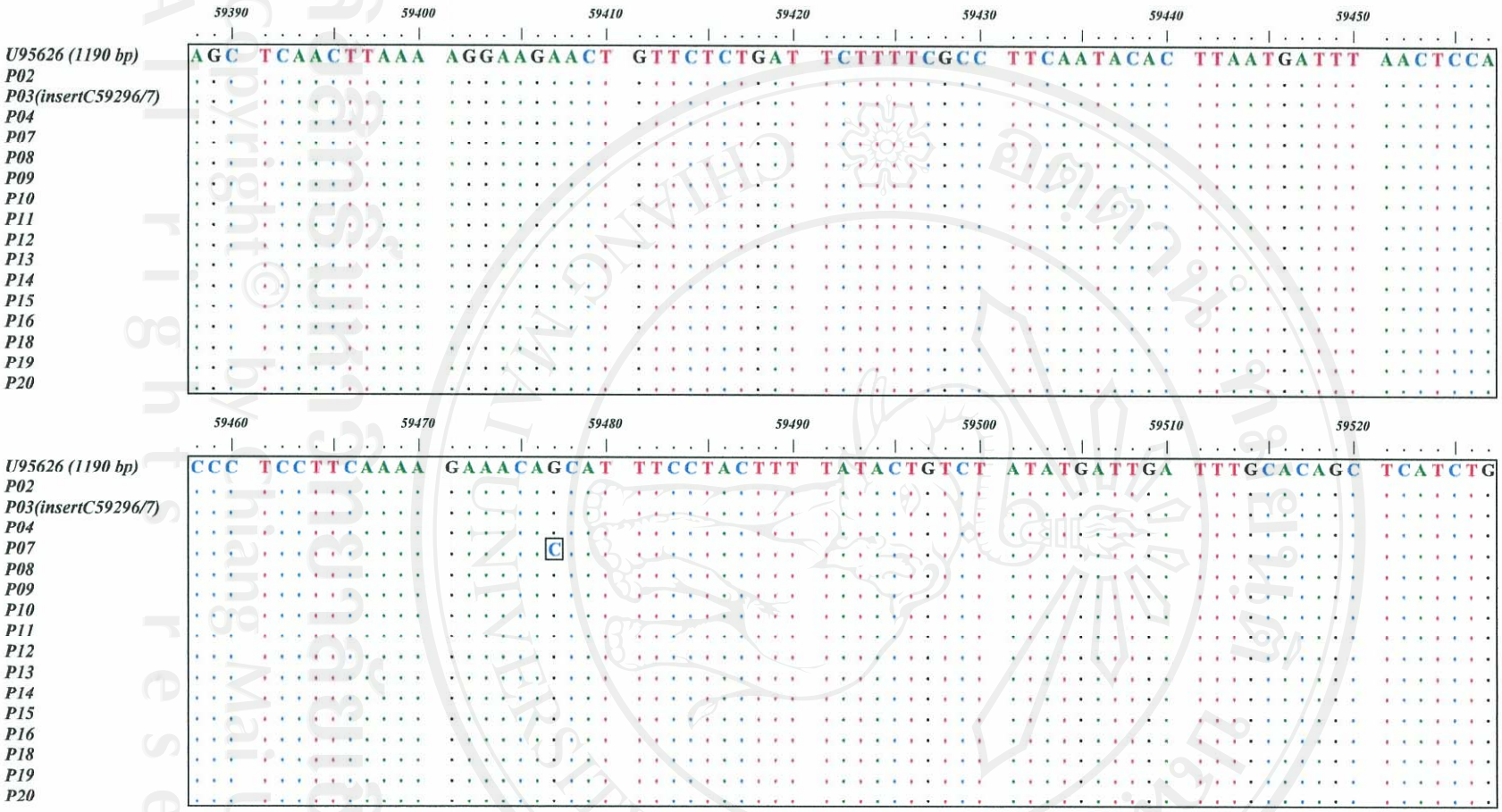
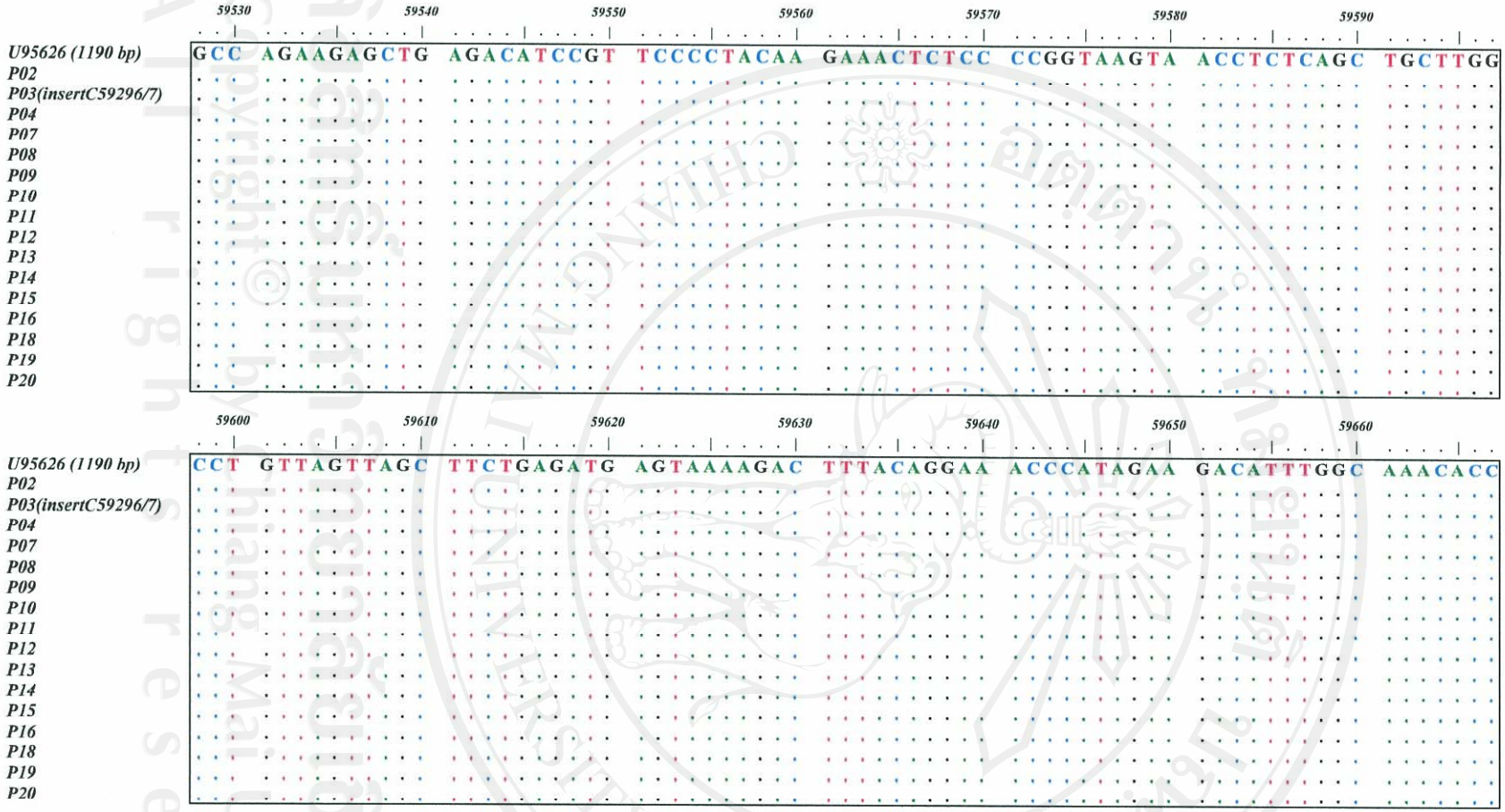
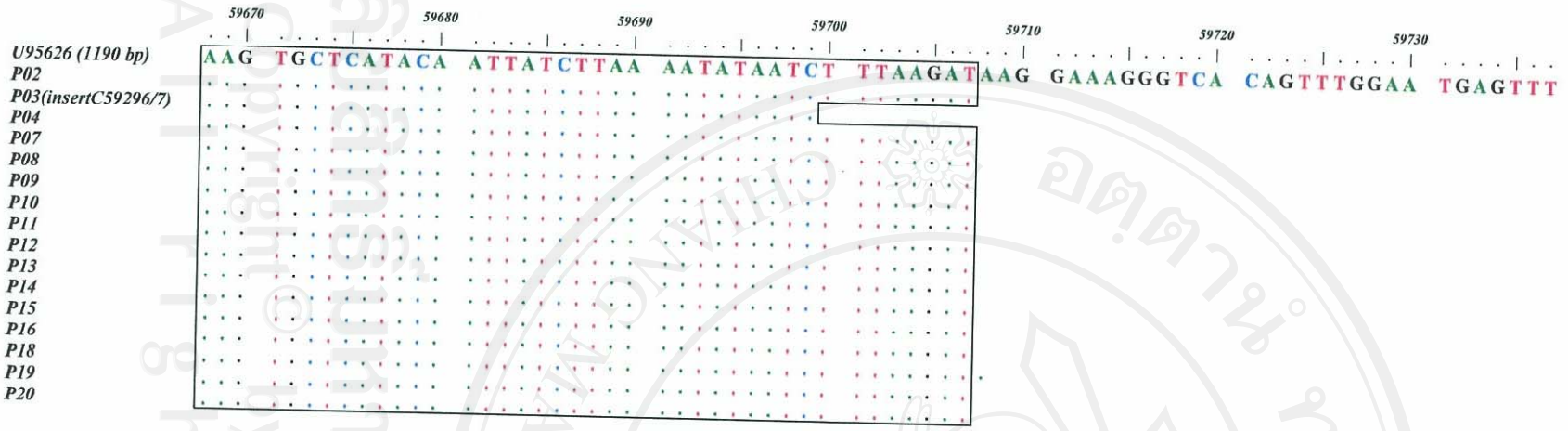
P02
P03(insertC59296/7)
P04
P07
P08
P09
P10
P11
P12
P13
P14
P15
P16
P18
P19
P20
59040
59050
59060
59070
59080
59090
59100
U95626 (1190 bp) TAA TGTGAAGTCC AGGATCCCCC TCTACATTTA AAGTTGGTTT AAGTTGGCTT TAATTAATAG CAACTCT
P02
P03(insertC59296/7)
P04
P07
P08
P09
P10
P11
P12
P13
P14
P15
P16
P18
P19
P20











U95626 (1190 bp)
P02
P03(insertC59296/7)
P04
P07
P08
P09
P10
P11
P12
P13
P14
P15
P16
P18
P19
P20

2.4 Analysis of the nucleotide polymorphisms of CCR5 promoter region

In this study, we screened for the mutation in the 1190 bp DNA fragment containing CCR5 promoter region from 19 HEPS (H02-H06 and H08-H20) and 16 HIV-1 seropositive spouses (P02-P04, P07-P16 and P18-P20). According to the 10 polymorphic nucleotide positions in CCR5 promoter region, 58,934, 59,338, 59,352, 59,353, 59,356, 59,373, 59,402, 59,410, 59,440, and 59,537 (number corresponding to GenBank accession number U95626), identified recently, by Martin *et al.* (1998) has classified into 10 CCR5 promoter haplotypes (P1-P10). We found that 18 of 19 samples from HEPS group were identified as CCR5P4 haplotypes with the nucleotide 58934T, 59353T and 59402G. They were all homozygous type, thus classified as the CCR5P4/P4 genotype. The only one sample from HEPS-H07 was defined as CCR5P2 haplotype with the nucleotide 58934G, 59353T and 59402A. It also was the CCR5P2/P2 genotype since the homozygosity of the alleles. Both CCR5 promoter haplotypes (P2 and P4) observed here are among the common haplotypes present worldwide. Addition to nucleotide positions described above, we also looking for the polymorphisms at other nucleotide positions that have been reported to have a clinical relevance in HIV-1 disease progression. Among those, CCR5-59029A/G, CCR5-59353C/T and CCR5-59653C/T polymorphisms (the first letter indicates the wild-type nucleotide and the second indicates the mutant nucleotide) have frequently found to associate with disease progression. We found here that all except one HEPS (H07) individuals have the homozygous mutant nucleotide G at position 59029 (59029G/G genotype) while the HEPS (H07) has a wild-type CCR5-59029A/A genotype. It is noted that the CCR5-59029G/G genotype was associated with the P4 haplotype while the CCR5-59029A/A associated with P2 haplotype. The mutant nucleotide T at the position 59353 was found in all samples from both HEPS and HIV-1 seropositive individuals. At the CCR5-59653 position, all except one sample from HEPS (H02) were wild-type homozygous 59653C/C while the only one HEPS (H02) was heterozygous 59653C/T. The other minor mutations were more frequently observed in the HEPS than the seropositive group. However, we did not see the significant different among the sequence of CCR5 promoter region from the HEPS and their HIV-1 seropositive spouses. The comparison of the multisite alleles of the CCR5 promoter gene of 19 HEPS subjects and 16 HIV-1 seropositive subjects with Martin *et al* (1998) are shown in Table 5 and 6, respectively.

Table 5 Comparison of the multisite alleles of the CCR5 promoter gene of 19 HEPS subjects with Martin *et al.* (1998)

Samples	Multisite alleles										allele haplotypes
	208[1]	612[1]	626[1]	627[1]	630[1]	647[1]	676[1]	684[1]	714[1]	811[1]	
	58934[2]	59338[2]	59352[2]	59353[2]	59356[2]	59373[2]	59402[2]	59410[2]	59440[2]	59537[2]	
H02	T	A	C	T	C	C	G	T	C	G	**P4**
H03	T	A	C	T	C	C	G	T	C	G	**P4**
H04	T	A	C	T	C	C	G	T	C	G	**P4**
H05	T	A	C	T	C	C	G	T	C	G	**P4**
H06	T	A	C	T	C	C	G	T	C	G	**P4**
H07	G	A	C	T	C	C	A	T	C	G	**P2**
H08	T	A	C	T	C	C	G	T	C	G	**P4**
H09	T	A	C	T	C	C	G	T	C	G	**P4**
H10	T	A	C	T	C	C	G	T	C	G	**P4**
H11	T	A	C	T	C	C	G	T	C	G	**P4**
H12	T	A	C	T	C	C	G	T	C	G	**P4**
H13	T	A	C	T	C	C	G	T	C	G	**P4**
H14	T	A	C	T	C	C	G	T	C	G	**P4**
H15	T	A	C	T	C	C	G	T	C	G	**P4**
H16	T	A	C	T	C	C	G	T	C	G	**P4**
H17	T	A	C	T	C	C	G	T	C	G	**P4**
H18	T	A	C	T	C	C	G	T	C	G	**P4**
H19	T	A	C	T	C	C	G	T	C	G	**P4**
H20	T	A	C	T	C	C	G	T	C	G	**P4**

[1] according to Martin *et al.*, 1998

[2] according to GenBank accession number U95626

Table 6 Comparison of the multisite alleles of the CCR5 promoter gene of 16 HIV-1 seropositive subjects with Martin *et al.* (1998)

Samples	Multisite alleles										allele haplotypes
	208[1]	612[1]	626[1]	627[1]	630[1]	647[1]	676[1]	684[1]	714[1]	811[1]	
	58934[2]	59338[2]	59352[2]	59353[2]	59356[2]	59373[2]	59402[2]	59410[2]	59440[2]	59537[2]	
P02	T	A	C	T	C	C	G	T	C	G	**P4**
P03	T	A	C	T	C	C	G	T	C	G	**P4**
P04	T	A	C	T	C	C	G	T	C	G	**P4**
P07	T	A	C	T	C	C	G	T	C	G	**P4**
P08	T	A	C	T	C	C	G	T	C	G	**P4**
P09	T	A	C	T	C	C	G	T	C	G	**P4**
P10	T	A	C	T	C	C	G	T	C	G	**P4**
P11	T	A	C	T	C	C	G	T	C	G	**P4**
P12	T	A	C	T	C	C	G	T	C	G	**P4**
P13	T	A	C	T	C	C	G	T	C	G	**P4**
P14	T	A	C	T	C	C	G	T	C	G	**P4**
P15	T	A	C	T	C	C	G	T	C	G	**P4**
P16	T	A	C	T	C	C	G	T	C	G	**P4**
P18	T	A	C	T	C	C	G	T	C	G	**P4**
P19	T	A	C	T	C	C	G	T	C	G	**P4**
P20	T	A	C	T	C	C	G	T	C	G	**P4**

[1] according to Martin *et al.*, 1998

[2] according to GenBank accession number U95626

3. Determination of the CCR5Δ32 genotype

Genomic DNA samples from 48 subjects (20 HEPS subjects, 18 HIV-1 seropositive subjects, and 10 normal control subjects) were amplified for the CCR5 coding region flanking with the 32 bp deletion region by PCR technique using R5-32F and R5-32R primers as reported by Quillent *et al*, 1998. The PCR product of the wild-type allele was a 198 bp, whereas the CCR5Δ32 allele was a 166 bp. The results shown in Figure 16 indicated the homozygous wild-type alleles in the samples. None of the CCR5Δ32 allele was detected amongst 48 DNA samples. All of them were shown to contain homozygous wild-type alleles. All negative controls were absolutely negative with those primers, which those primers indicating the absence of contamination in this experiment.

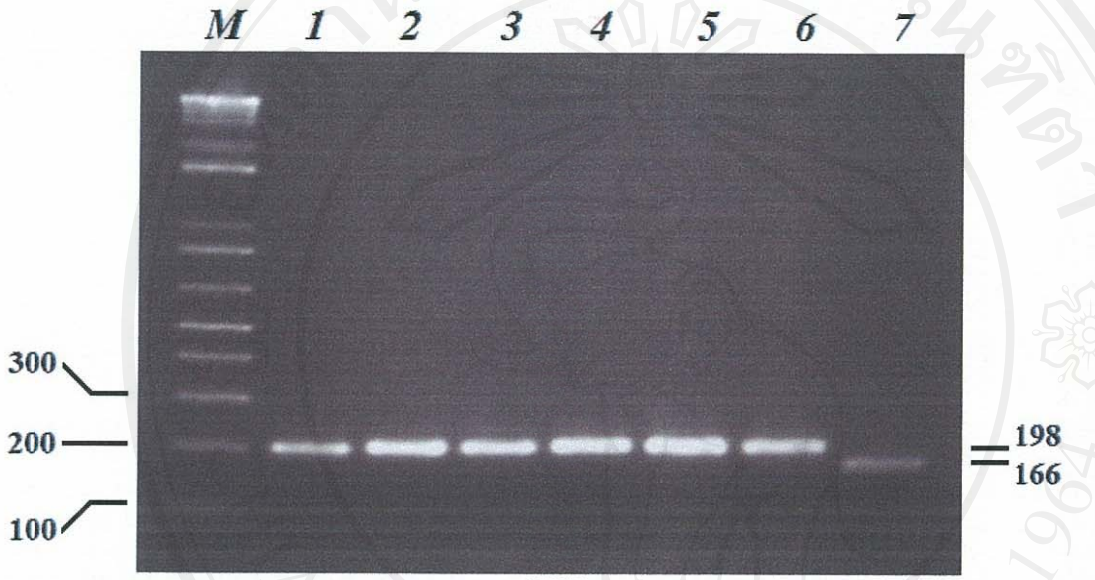


Figure 16 PCR amplification of the CCR5 ORF by specific primers flanking with the CCR5Δ32 region. A 1 Kb Plus DNA Ladder was used as a marker (lane *M*). Lane *1* and *2* show the PCR product from HEPS subjects (H01 and H02), lane *3* and *4* show the PCR product from HIV-1 seropositive subjects (P01 and P02), lane *5* and *6* show the PCR product from normal control subjects (N01 and N02), and lane *7* shows the PCR product of a homozygous 32 bp deletion (CCR5Δ32/Δ32).

4. Determination of the CCR5-m303 genotype

Genomic DNA samples from 48 subjects (20 HEPS subjects, 18 HIV-1 seropositive subjects, and 10 normal control subjects) were amplified for the CCR5 coding region flanking with the m303 site by Nested-PCR using a set of outer primers (R5-303F and R5-303R primers) as reported by Quillent *et al*, 1998 and a set of inner primers (R5-303NF and R5-303NR primers) which was designed and used in our laboratory. The first round PCR product was approximate 1,076 bp (Figure 17), while the second round PCR produced a band of approximate 737 bp (Figure 18). Each of the second round PCR products from all subjects was then detected for a single point mutation at position 303 by using restriction endonuclease *Hinc*II. All negative controls were absolutely negative with those primers. After digestion, the DNA fragments of the wild-type allele were digested into 2 fragments approximately 528 and 209 bp, whereas the CCR5-m303 allele was not digested (Figure 19). The results showed that no CCR5-m303 was detected in these samples. They were all homozygous wild-type alleles.

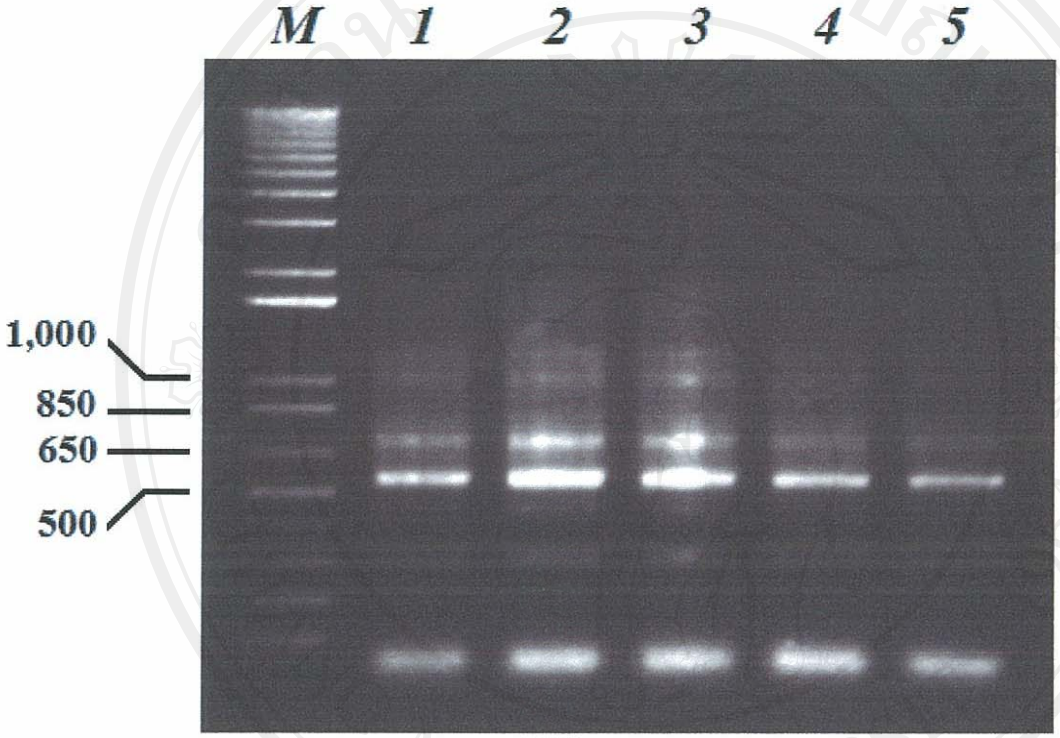


Figure 17 First round amplification of the CCR5 ORF by R5-303F and R5-303R primers. Lane *M* shows 1 Kb Plus DNA Ladder marker. Lane *1* and *2* show the PCR product from HEPS subjects (H01 and H02), lane *3* and *4* show the PCR product from HIV-1 seropositive subjects (P01 and P02), and lane *5* shows the PCR product from normal control subject (N01).

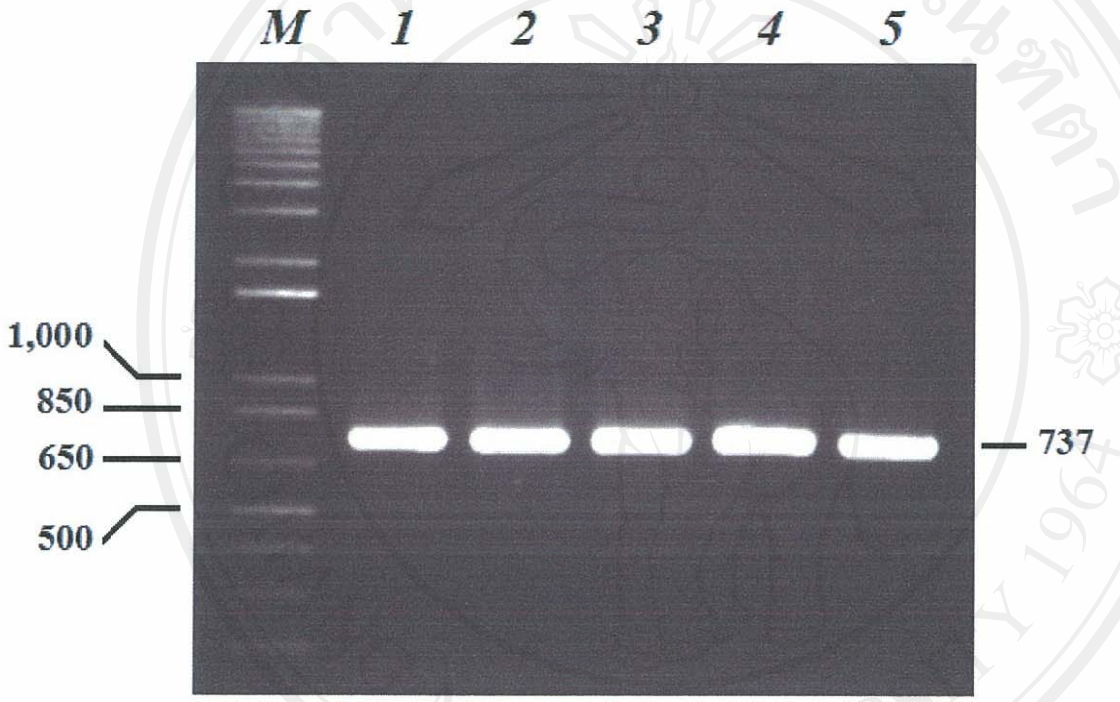


Figure 18 Second round amplification of the CCR5 ORF by R5-303NF and R5-303NR primers. Lane ***M*** shows 1 Kb Plus DNA Ladder marker. Lane ***1*** and ***2*** show the PCR product from HEPS subjects (H01 and H02), lane ***3*** and ***4*** show the PCR product from HIV-1 seropositive subjects (P01 and P02), and lane ***5*** shows the PCR product from normal control subject (N01).

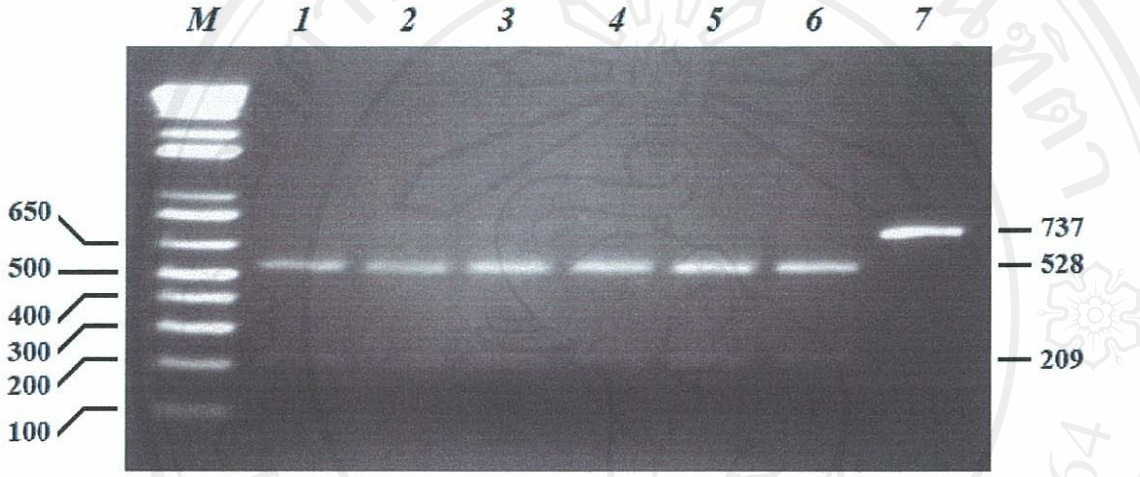


Figure 19 Detection of the CCR5-m303 genotype by cleavage of a 737 bp PCR product with *Hinc*II restriction endonuclease. Lane ***M*** shows 1 Kb Plus DNA Ladder marker. Lane ***1*** and ***2*** show the digested fragments from HEPS subjects (H01 and H02), lane ***3*** and ***4*** show the digested fragments from HIV-1 seropositive subjects (P01 and P02), and lane ***5*** and ***6*** show the digested fragments from normal control subjects (N01 and N02). Lane *7* shows the 737 bp DNA segment as refer to the m303 allele.

5. Determination of the CCR5 protein density on surface of CD4+ T lymphocytes and monocytes

Fresh EDTA whole blood from the HIV-1 serodiscordant group and the normal control group were used to determine the expression of CCR5 molecule on CD4+ lymphocytes and monocytes (CD14+). At the time of this experiment, some of the blood samples were not possible to collected from some individuals in the serodiscordant group (H08, H09, H12, H20, P04, P06, P07, P08, P09, P12, and P20).

5.1 Expression of the membrane CCR5 on CD4+ T lymphocytes

Fresh EDTA whole blood from the HIV-1 serodiscordant group and the normal control group (n = 36: 16 HEPS subjects, 10 HIV-1 seropositive subjects, and 10 normal subjects) were determined for the surface expression of CCR5 on CD4+ T lymphocytes by the direct immunofluorescent technique and flow cytometry using PE-conjugated IgG2a isotype control, PE-conjugated anti-CCR5 mAb and FITC-conjugated anti-CD4 mAb (Figure 20). The results showed that the expression varied among individuals, with the percentages of CCR5+CD4+ cells in total lymphocytes of HEPS subjects ranging from 3.17 to 13.86% (mean ± SD = 6.79 ± 3.23, median = 5.74), HIV-1 seropositive subjects ranging from 0.85 to 5.62% (mean ± SD = 3.11 ± 1.54, median = 2.99), and normal subjects ranging from 4.22 to 8.78% (mean ± SD = 6.29 ± 1.63, median = 6.22). Comparison of CCR5 expression between HEPS and their seropositive spouses showed that the HIV-1 seropositive spouses had significantly lower percentages of the CCR5+CD4+ cells in total lymphocytes ($p = 0.01$), whereas the percentages of these cells was not significantly difference between HEPS and normal groups ($p = 0.66$). Both HEPS and healthy normal groups had significantly higher percentages than those obtained from HIV-1 seropositive group (p=0.01 and p=0.001, respectively) (Figure 21). Additionally, a population of CCR5+CD4- cells was showed in the upper left quadrant of each stain of Figure 20 indicated that both CD4+ and CD4- T lymphocyte populations had the expression of the membrane CCR5.

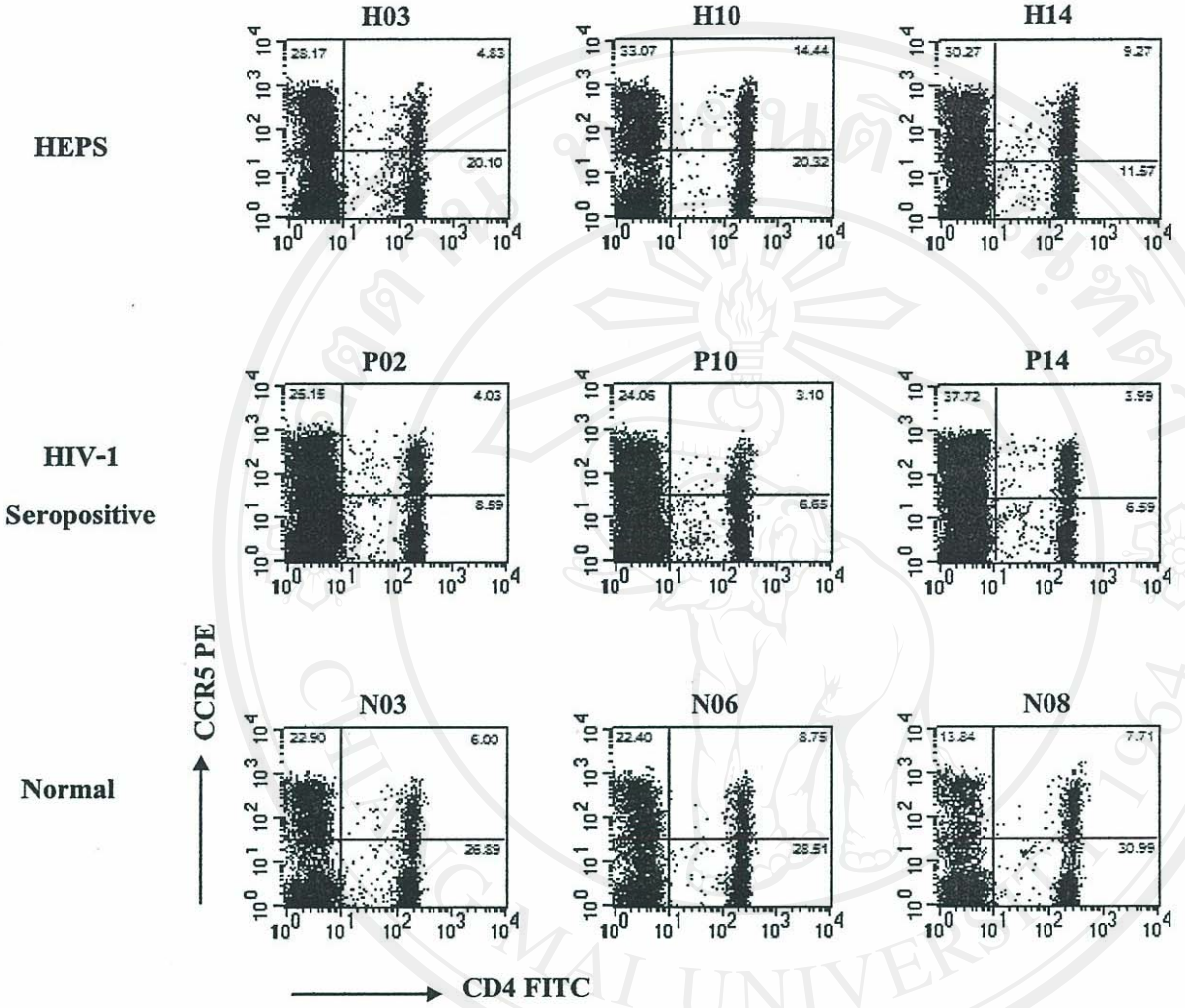


Figure 20 CCR5 expression on CD4+ T lymphocytes of HEPS subjects (H03, H10, and H14), HIV-1 seropositive subjects (P02, P10, and P14), and normal subjects (N03, N06, and N08) as analyzed by flow cytometry using anti-CCR5 PE and anti-CD4 FITC. The numbers show the percentages of cells in each quadrant. The number in the upper right quadrant is the percentage of CCR5 positive cells in total CD4 positive (CCR5+CD4+).

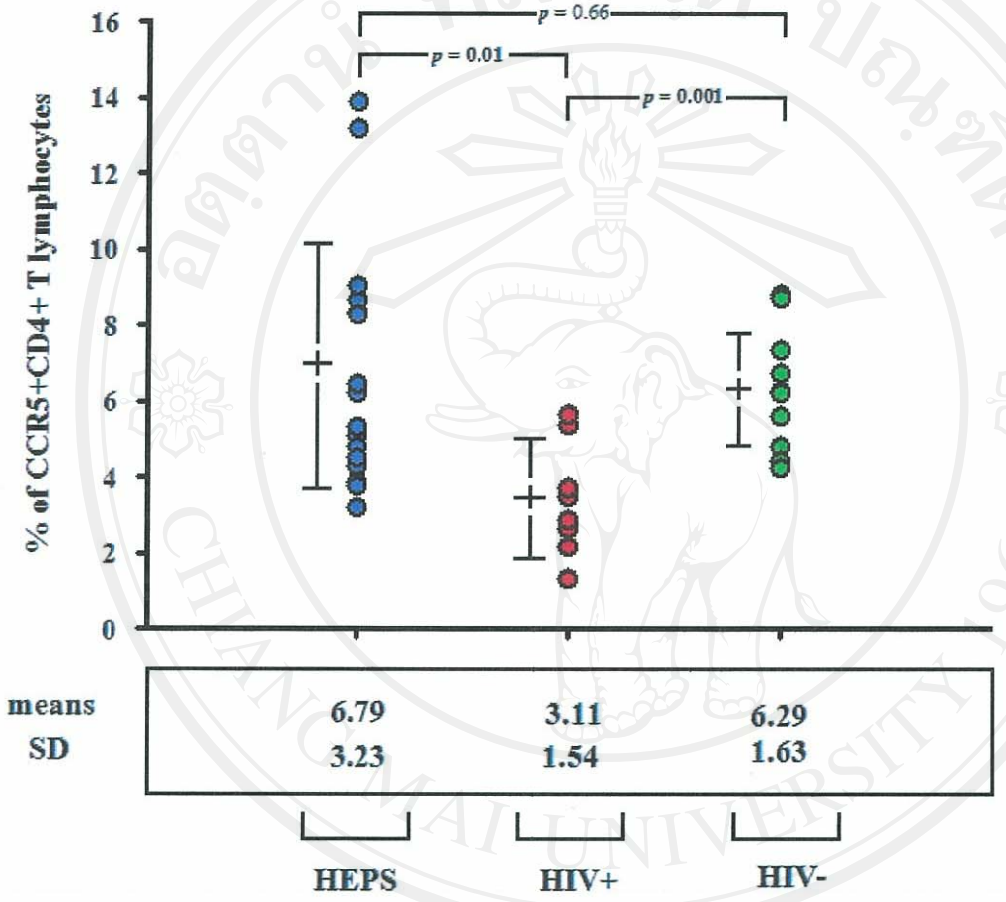


Figure 21 Percentage of CCR5+CD4+ cells in total lymphocytes population from (●) HEPS, (●) HIV-1 seropositive, and (●) normal subjects. The lines represent means with SD. The numbers under the plots are the mean percentage and SD of CCR5+CD4+ cells in each study subjects.

5.2 Expression of the membrane CCR5 on monocytes

The expression of CCR5 on monocytes (CD14+) was also determined in all three groups of the study subjects (N = 36; 16 HEPS individuals, 10 HIV-1 seropositive individuals, and 10 HIV-1 seronegative individuals). As expected, the similarity observed in the surface expression between CD4+ T lymphocytes and CD14+ monocytes populations was the variety of the expression among individuals (Figure 22). The percentages of CCR5+ on CD14+ cells in total monocytes of HEPS subjects ranging from 2.93 to 32.99% (mean ± SD = 17.41 ± 9.22, median = 15.52), HIV-1 seropositive subjects ranging from 10.62 to 30.80% (mean ± SD = 21.30 ± 6.77, median = 22.97), and normal control subjects ranging from 0.71 to 13.43% (mean ± SD = 7.49 ± 3.69, median = 7.39). The difference in CCR5 expression between CD4+ T lymphocytes and CD14+ monocytes populations was also compared of the expression between each group of subjects. The results showed that the percentage of CCR5+CD14+ cells of normal control subjects was significantly lower than these cells obtained from both HEPS and HIV-1 seropositive subjects (p = 0.004 and p = 0.00002, respectively), but percentages of those cells from HEPS and HIV-1 seropositive subjects were not significantly different (p = 0.26) (Figure 23). In addition, the pattern of CCR5 expression on surface monocytes as showed in Figure 22 indicated that every cell in the monocytes population had the expression of the membrane CCR5.

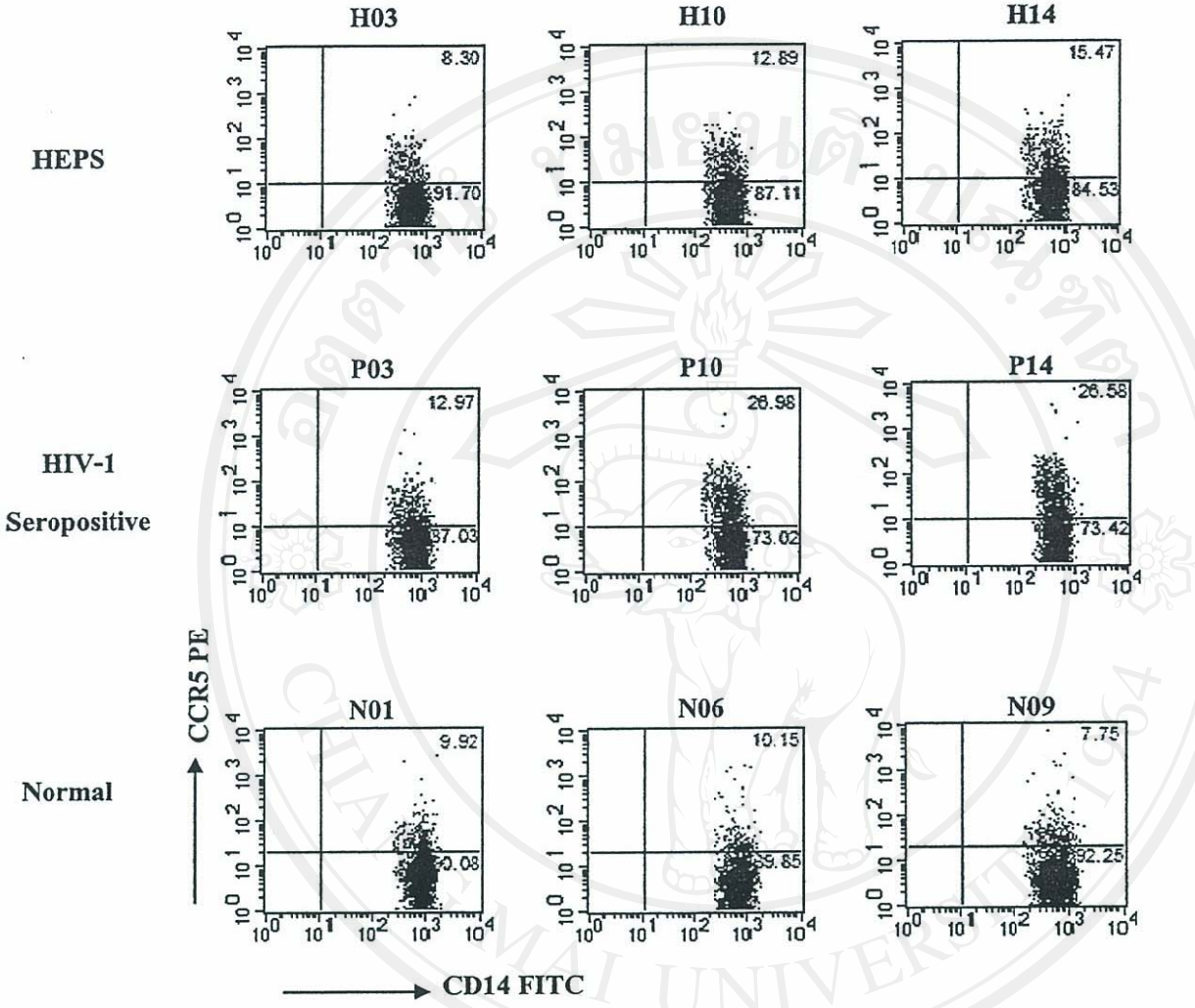


Figure 22 CCR5 expression on monocytes (CD14+) of HEPS subjects (H03, H10, and H14), HIV-1 seropositive subjects (P03, P10, and P14), and normal control subjects (N03, N06, and N08) as analyzed by flow cytometry using anti-CCR5 PE and anti-CD14 FITC. The numbers show the percentages of cells in each quadrant. The number in the upper right quadrant is the percentage of CCR5 positive cells in total CD14 positive (CCR5+CD14+).

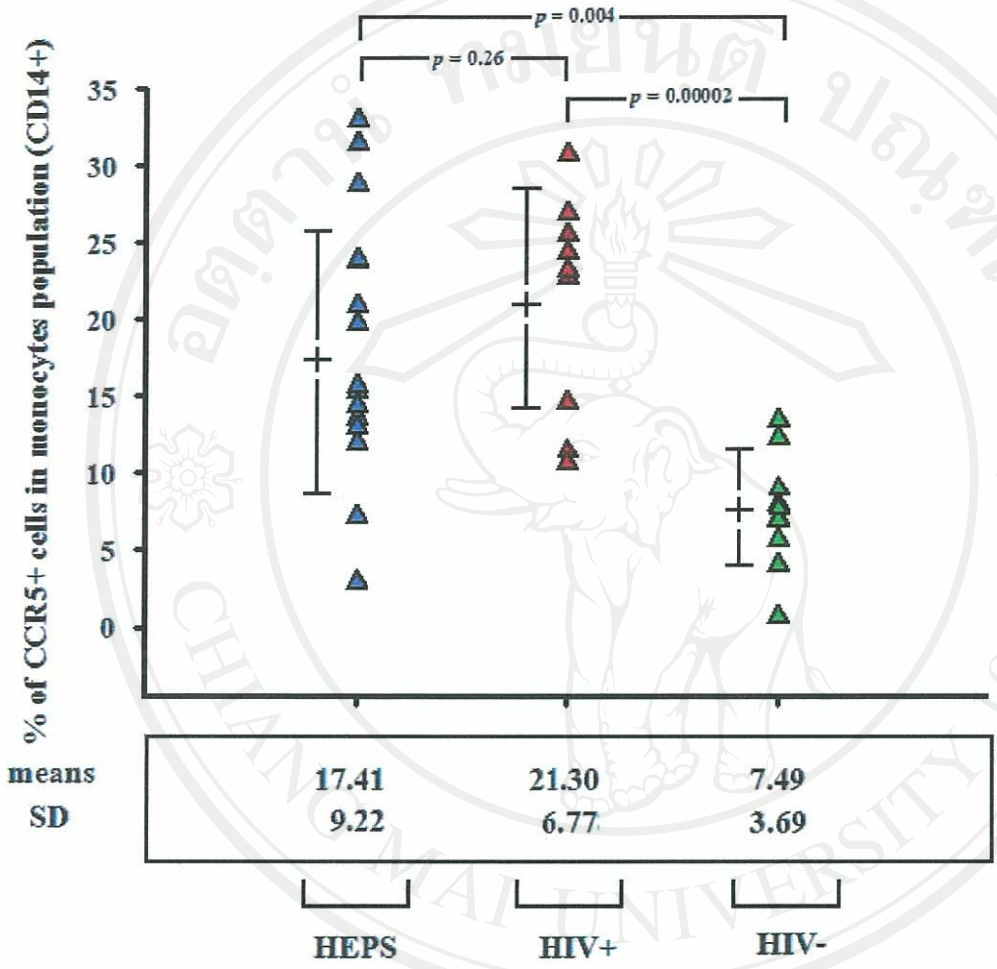
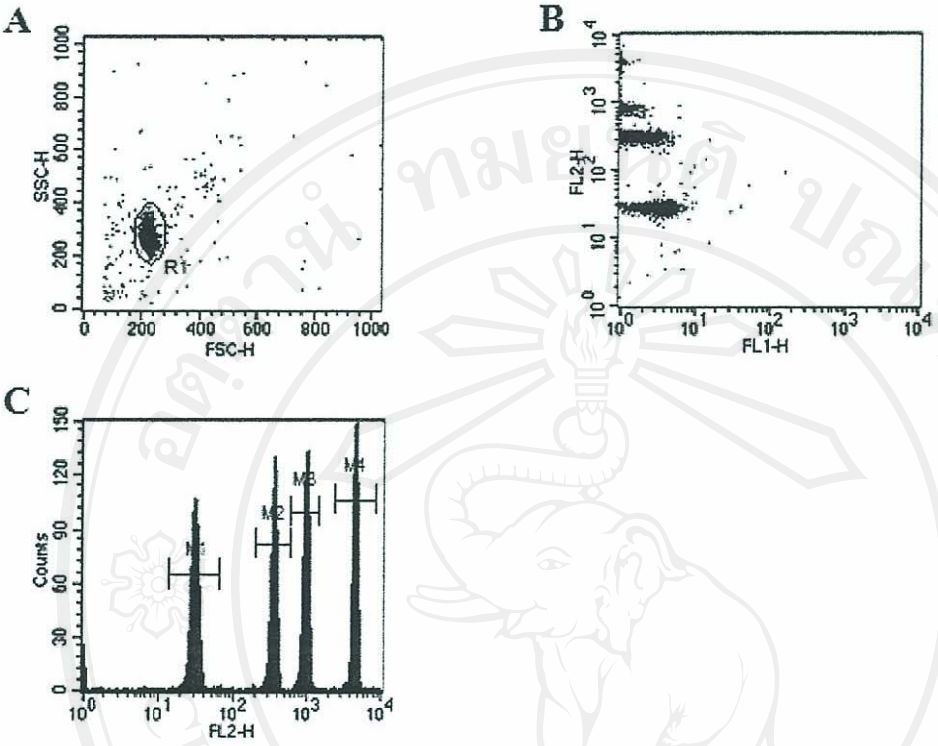


Figure 23 Percentage of CCR5+ on CD14+ cells in total monocytes from (▲) HEPS subjects, (▲) HIV-1 seropositive subjects, and (▲) normal control subjects. The lines represent means with SD. The numbers under the plots are the mean percentage and SD of CCR5+CD14+ cells in each study subjects.

5.3 Calibration curve for determination of the CCR5 protein density

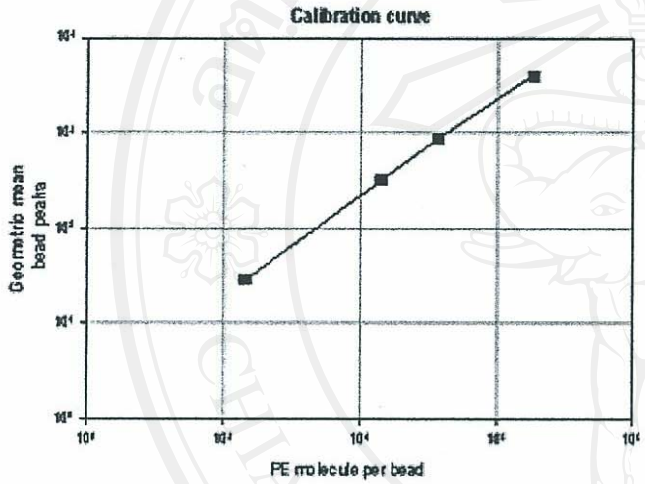
QuantiBRITE™PE (the Phycoerythrin Fluorescence Ouantitation Kit) was used to estimate the antibodies bound per cell in the flow cytometric analysis. The bead singlets would be obtained after running for the QuantiBRITE PE tube by CellQuest software collected exactly 10,000 events, and then gating around the population of the bead singlets on FSC-H vs. SSC-H plot (Figure 24A). The singlet bead population, which composed of 4 sets of PE-conjugated microbeads (Figure 24B), was then analyzed using a histogram plot of FL2-H in linear values (Figure 24C). The geometric means created from the fluorescence intensities of the four bead peaks obtained form the histogram statistics window (Figure 24D) were used to construct a calibration curve (mean fluorescence intensity against number of PE molecules per bead) (Figure 25).



D

Marker	Events	% Gated	% Tota	Mean	Geo Mean	SD	CV	Median
All	10000	100.00	100.00	1271.41	389.27	1590.64	125.11	358.66
M1	2519	25.19	25.19	28.47	28.25	3.67	12.88	28.39
M2	2577	25.77	25.77	321.79	320.21	32.16	9.99	321.97
M3	2358	23.58	23.58	869.64	867.02	67.43	7.75	873.79
M4	2426	24.26	24.26	4010.96	3998.89	327.70	8.17	4081.52

Figure 24 Determination of the QuantiBRITE™PE standard quantitation beads by CellQuest software; **A** shows gating around the population of the bead singlets on FSC-H vs. SSC-H plot; **B** shows four populations of PE-conjugated microbeads on FL1-H vs. FL2-H plot; **C** shows four populations of PE-conjugated microbeads analyzed using a histogram plot of FL2-H in linear values; **D** shows details and geometric means fluorescence intensities of the four bead peaks analyzed by CellQuest software.



PE molecules per bead from package insert	Geometric means of bead peaks (linear fluorescence)
1400	28.3
14000	320.2
36600	867.0
182000	3998.9

Figure 25 Calibration curve. The calibration curve obtained from the geometric means fluorescence intensities of the four bead peaks (mean fluorescence intensity against number of PE molecules per bead).

5.4 CCR5 density on surface of CD4+ lymphocytes

After subtraction of the geometric means fluorescence of the isotype control from the geometric means fluorescence of the CCR5-PE positive cells, the CCR5 protein density at the surface of CD4+ T lymphocytes of each subject was calculated by converting the subtracted geometric means fluorescence into the number of antibodies bound per cell (the membrane CCR5 molecules per cell (molecules/cell)) by interpolation on the equation of calibration curve using QuantiCal software (Becton Dickinson; CA, USA). The samples of immunofluoresences profile of the CCR5 expression on CD4+ T lymphocytes is shown in Figure 26. The CCR5 density on surface of CD4+ lymphocytes of HEPS subjects ranging from 1 to 257 molecules/cell (mean ± SD = 99.07 ± 82.13), HIV-1 seropositive subjects ranging from 50 to 421 molecules/cell (mean ± SD = 194.4 ± 122.19), and normal control subjects ranging from 12 to 217 molecules/cell (mean ± SD = 96.60 ± 74.94). The difference in CCR5 density on surface of CD4+ lymphocytes between each group of subjects was compared of the expression. The results showed that the CCR5 density on surface of CD4+ lymphocytes of HIV-1 seropositive subjects had significantly higher than those from HEPS and normal control subjects ($p = 0.03$ and $p = 0.04$, respectively) (Figure 27).

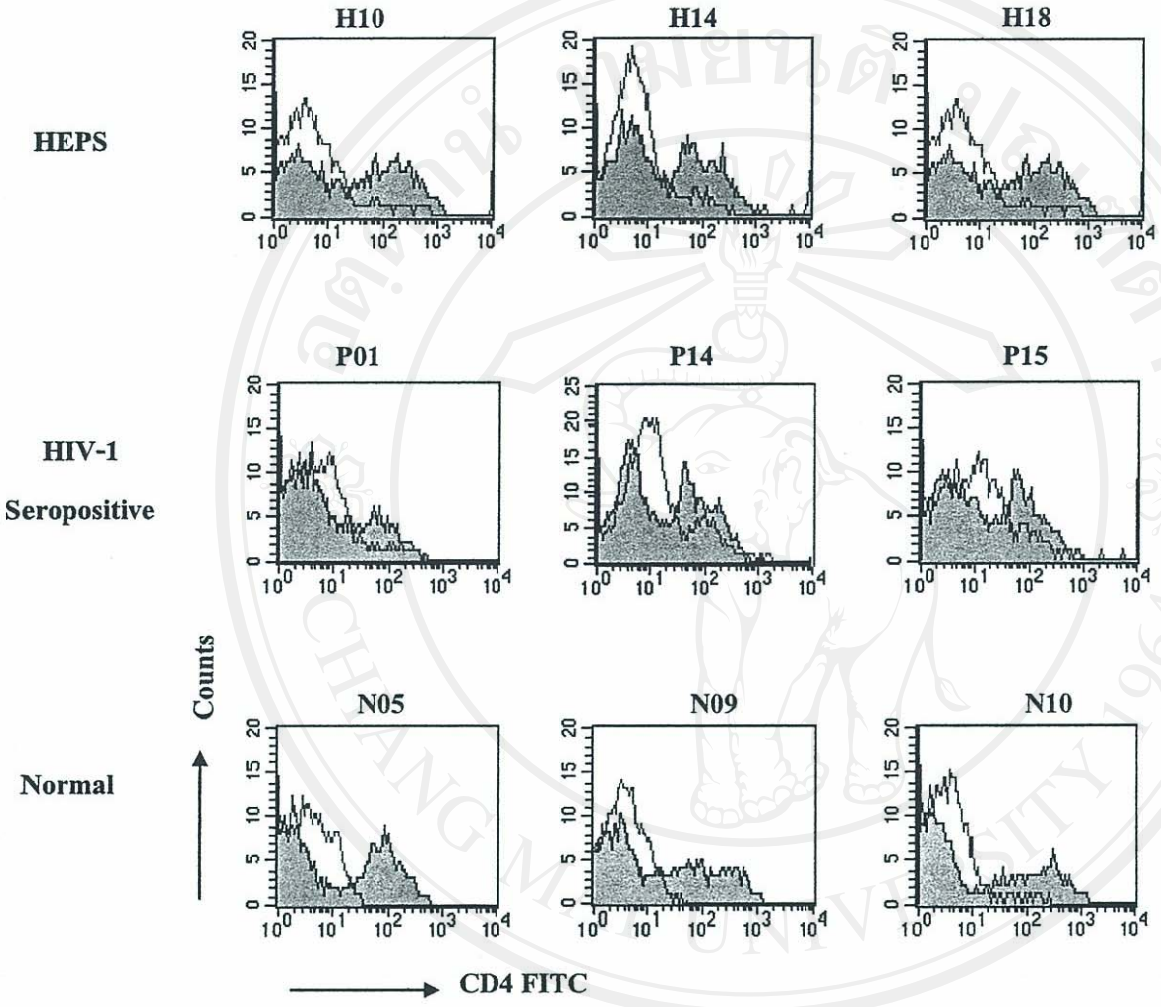


Figure 26 Fluorescence intensity of the CD4+ T lymphocytes expressed CCR5 molecule of HEPS subjects (H10, H14, and H18), HIV-1 seropositive subjects (P01, P14, and P15), and normal control subjects (N05, N09, and N10) as analyzed by flow cytometry using anti-CCR5 PE and anti-CD4 FITC.

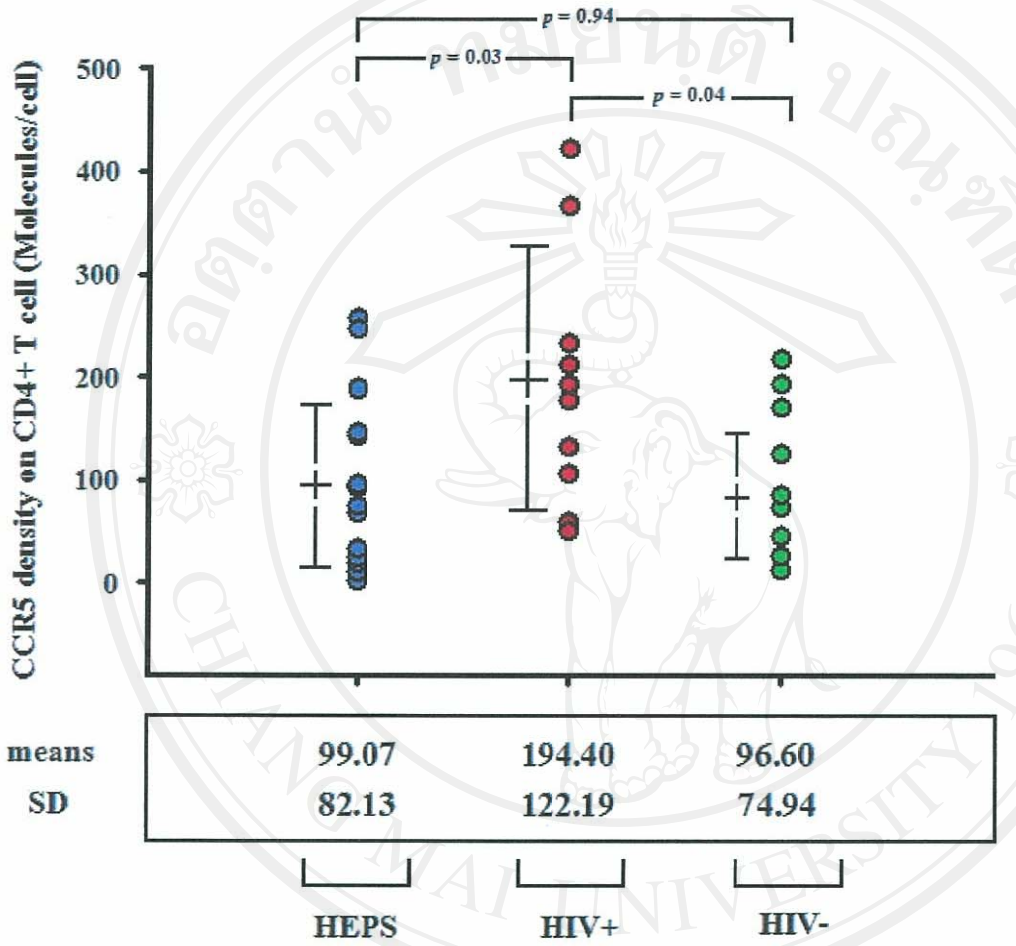


Figure 27 CCR5 density on CD4+ T lymphocytes from (●) HEPS, (●) HIV-1 seropositive, and (●) normal control subjects. The lines represent means with SD. The numbers under the plots are the mean percentage and SD of CCR5 density on CD4+ T lymphocytes in each study subjects.

5.5 CCR5 density on surface of monocytes

The number of antibodies bound per cell (the membrane CCR5 molecules per cell (molecules/cell)) was calculated by subtracted the geometric means fluorescence intensity of the CCR5-PE positive cells with the geometric means fluorescence of the isotype control. Then the geometric means were interpolated on the equation of calibration curve using QuantiCal software. The samples of immunofluoresences profile of the CCR5 expression on monocytes is shown in Figure 28. The CCR5 density on surface of monocytes of HEPS subjects ranging from 25 to 273 molecules/cell (mean ± SD = 139.69 ± 77.04), HIV-1 seropositive subjects ranging from 65 to 400 molecules/cell (mean ± SD = 186.5 ± 101.76), and normal control subjects ranging from 14 to 179 molecules/cell (mean ± SD = 84 ± 47.52). The difference in CCR5 density on surface of monocytes between each group of subjects was compared. The results showed that the CCR5 density on surface of monocytes of HIV-1 seropositive subjects had significantly higher than those from normal control subjects ($p = 0.009$), but not significantly higher than those from HEPS subjects ($p = 0.19$) (Figure 29).

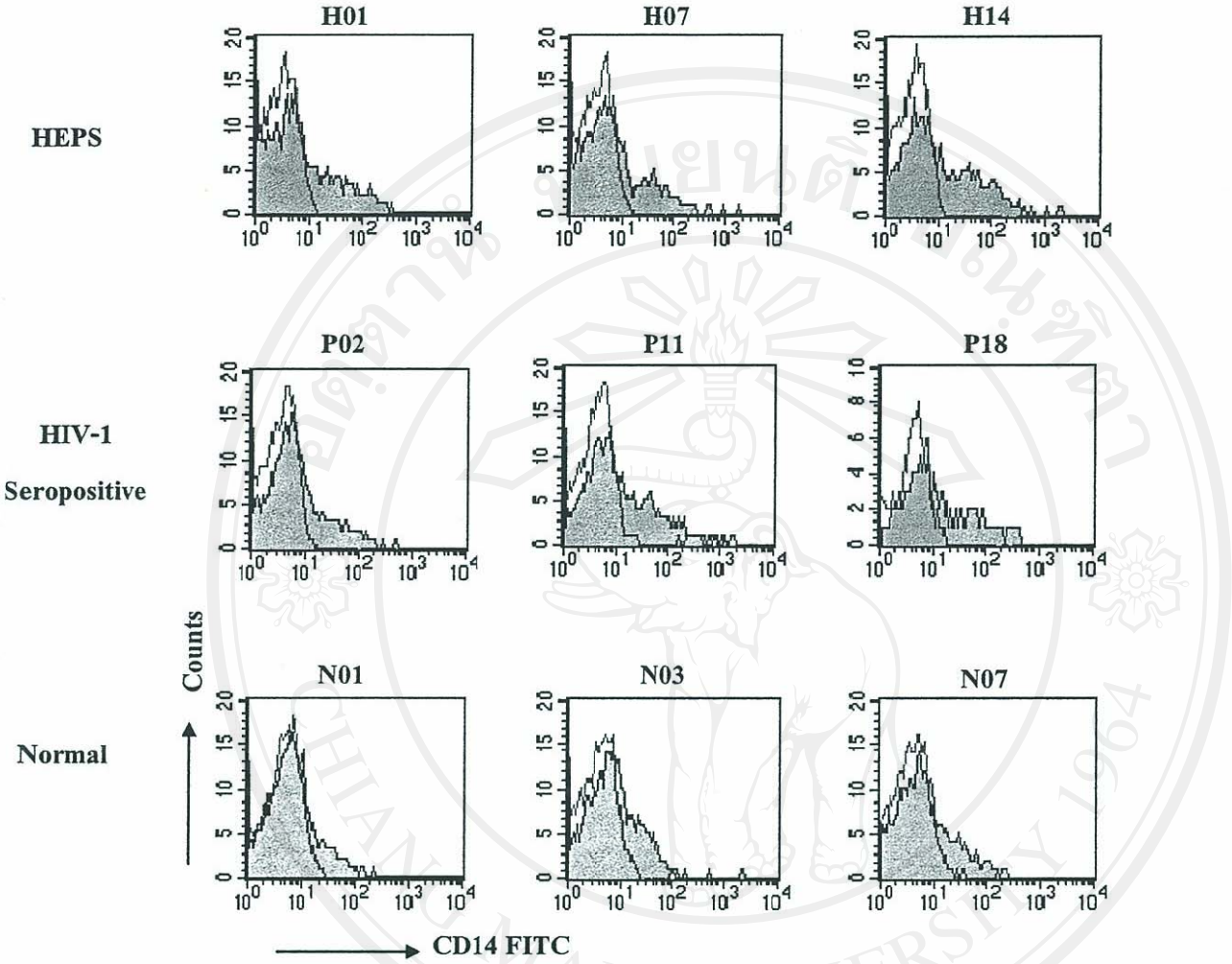


Figure 28 Fluorescence intensity of the monocytes expressed CCR5 molecule of HEPS subjects (H01, H07, and H14), HIV-1 seropositive subjects (P02, P11, and P18), and normal control subjects (N01, N03, and N07) as analyzed by flow cytometry using anti-CCR5 PE and anti-CD14 FITC.

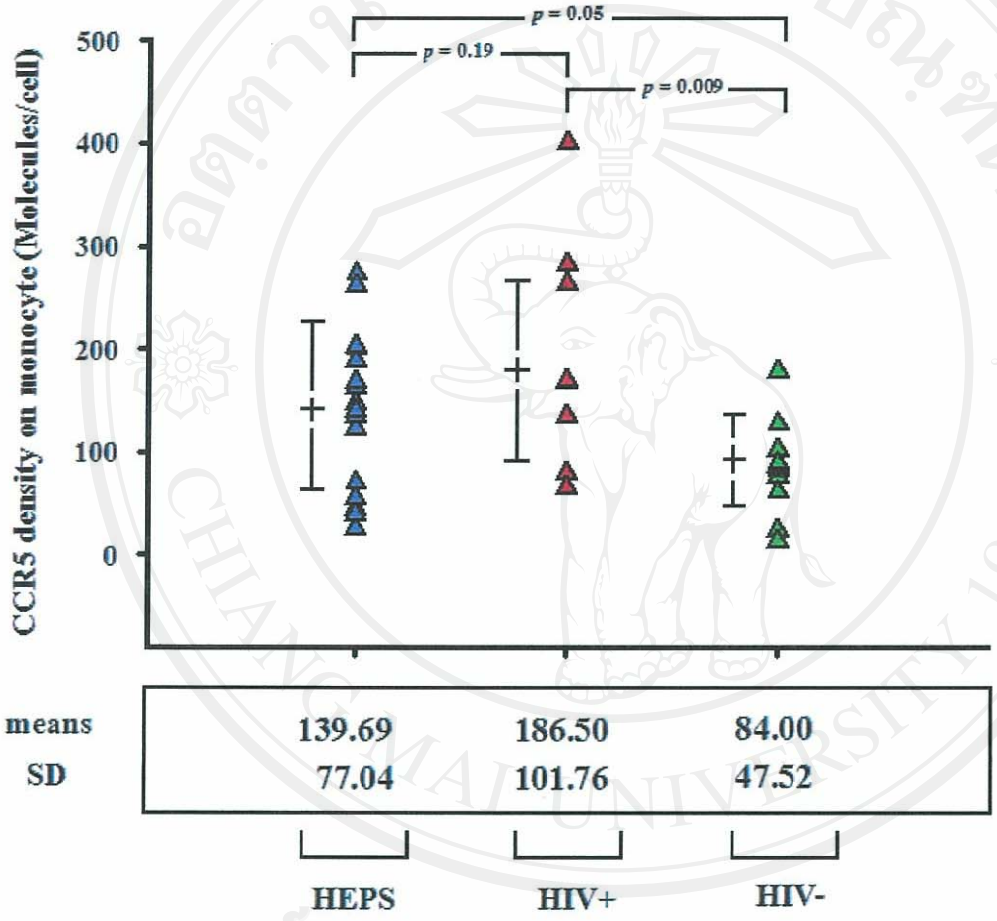


Figure 29 CCR5 density on monocytes from (●) HEPS, (●) HIV-1 seropositive, and (●) normal control subjects. The lines represent means with SD. The numbers under the plots are the mean percentage and SD of CCR5 density on monocytes in each study subjects.