CHAPTER V

RESULTS

5.1 Specimens for oligonucleotide ligation assay (OLA)

Plasma specimens (N = 40) of HIV-1 infected patients diagnosed as treatment failure (focusing on M184V mutation) at the Maharaj Nakorn Chiang Mai Hospital during 2007 to 2009 were used for performance determination of OLA. The demographic characteristics including HIV-1 M184V mutation data of clinical specimens were shown in Table 4.

Table 4 Demographic characteristics of HIV-1 infected patients with treatment failure and HIV-1 M184V mutation data of 40 clinical specimens.

Characteristic	Value (n=40)
Sex, n (%)	
Male	20 (50%)
Female	20 (50%)
HIV subtype, n (%)	
CRF01_AE	39 (97.5%)
B	1 (2.5%)
Median CD4 count, cell/mm3 [IQR]	180 [62 - 288]
Median HIV-1 RNA level, log copies/ml [IQR]	4.20 [3.80 – 4.81]
Median duration of ART, month [IQR]	51 [36-63]
HIV-1 M184V mutation, n (%)	
Wild-type (184M)	7 (17.5%)
Mutant (184V)	30 (75%)
Mix of wild-type and mutant	1 (2.5%)
Other mutation; M184I	2 (5%)

5.2 OLA primers and probes for M184V detection

The primers for the *pol* region of HIV-1 circulating in Thailand were developed according to the previously described OLA with some modifications (Beck et al., 2008). These primers were designed based on multiple sequences of HIV-1 subtype CRF01_AE (N = 101) which were retrieved from the Los Alamos National Laboratory website (lanl Database, http:// www. hiv. lanl. gov) (accessed date 6.10.2007). The modifications of primers were made by incorporating degenerate bases to accommodate polymorphisms encountered in the target sequences obtained from the CRF01_AE database. Whereas, the M184V probes specific for wild-type (184M) and mutant variants (184V) were designed based on multiple sequences alignment of the public HIV-1 sequence database (accessed via the internet with accessed date 6.10.2007) together with our own sequence database (accessed in 2007) of antiretroviral treatment failure patients. In order to accommodate the polymorphisms that usually occur in the HIV-1 genome, the base degeneracy at the polymorphism position was applied into the probe designing.

The 184M-wild-type and the 184V-mutant variants can be distinguishable by the ligation between the detector probe, for the wild-type or the mutant, and the common probe. The detector probes, for wild-type named “WT_184” and mutant named “MT_184V” and “Mod_MT184V” , are differ only one or two bases at the 3’ end and also were labeled at the 5’end with the digoxigenin and the fluorescein (Operon Biotechnologies GmbH, Cologne, Germany), respectively. Both probes hybridize with the target in the PCR product at the upstream direction from the base substitution position. Whereas, the common probe named “COMMON_184”, hybridize with the target at the downstream position adjacent to the detector probes

was biotinylated at the 3’ end and phosphorylated at the 5’ end (Bio Basic, Inc., Ontario, Canada). The specific probes for detecting the wild-type (184M) and mutant variants (184V) of HIV-1 circulating in the Thai population are shown in Table 5.

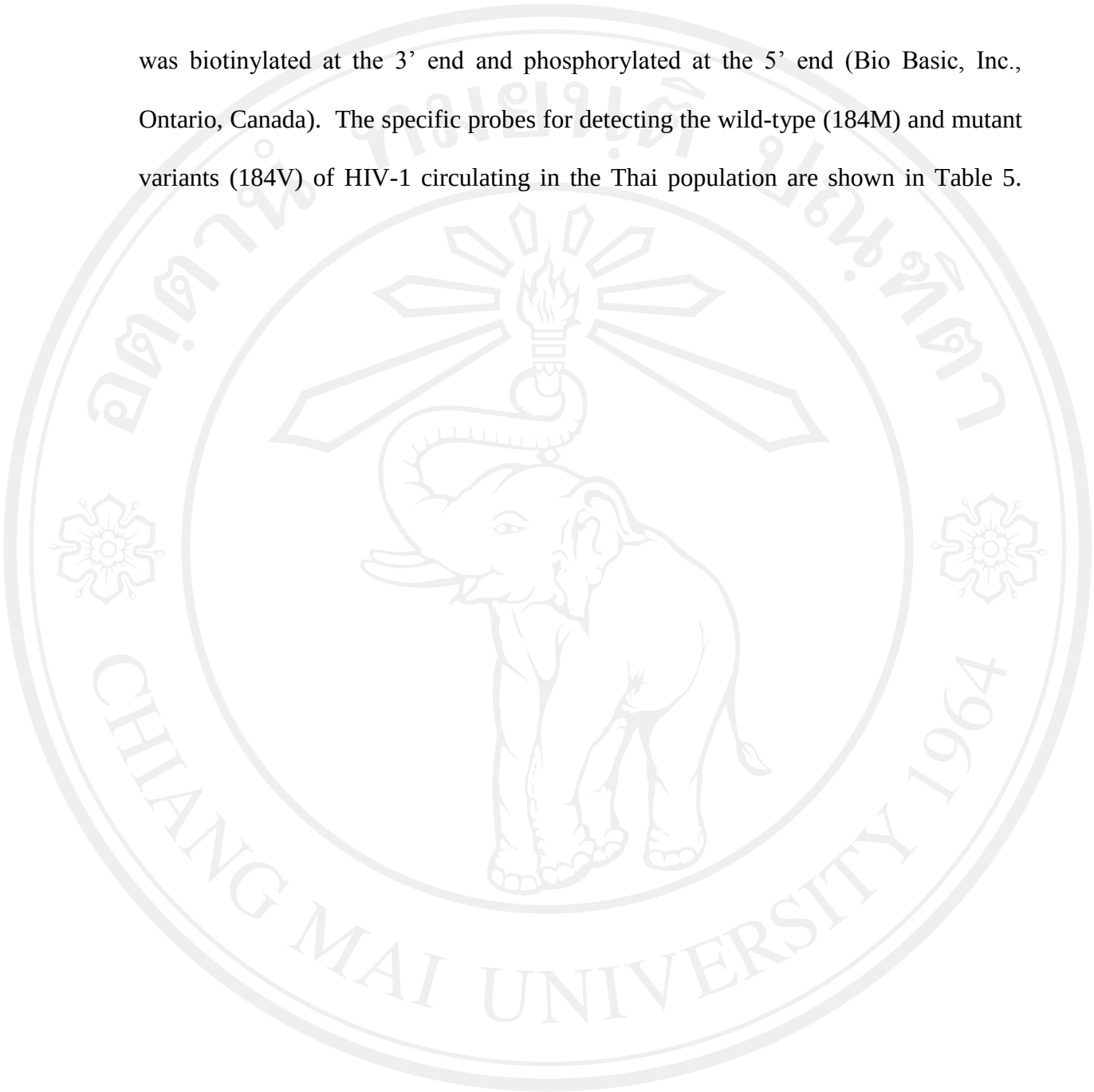

Table 5 Primers and probes for M184V detection

Primer/probe	Sequence (5'→ 3')	Position[a]	Application
UU1L_pol	5' CCT AGR AAA AAG GGY TGT TGG AAA TGT GG 3'	2011-2039	Outer primer
UU1R_pol	5' AAY TTY TGT AT G TCA TYG ACA GTC CA 3'	3303-3328	Outer primer
UU2L_pol	5' ACT GAR AGA CAG GCT AAT TTT TTA GGG A 3'	2068-2095	Inner primer
UU2R_pol	5' CAT YTG TCA GGR TGG AGT TCA TA 3'	3243-3265	Inner primer
WT_184	Dig-A GAA ATR GTT ATC TAT CAA TAC A	3077-3099	Wild type detector
MT_184V	Flu-A GAA ATR GTT ATC TAT CAA TAC G	3077-3099	Mutant detector
Mod_MT184V	Flu-A GAA ATR GTT ATC TAT CAA TCC G	3077-3099	Mutant detector
COMMON_184	p-TG GAT GAC TTG TAT GTA GGA TC-Bio	3100-3021	Common probe

[a] nucleotide position according to HXB2 numbering system
Dig = Digoxigenin Flu = Fluorescein p = Phosphate group Bio = Biotin

5.3 Preparation of specimens for OLA

HIV-1 RNA was extracted from plasma specimens by using QIAamp® Viral RNA Mini Kit (QIAGEN, Hilden, Germany). RNA was reverse transcribed to complementary DNA (cDNA), then amplified by using reverse transcription – polymerase chain reaction (RT-PCR) using the outer primers UU1L_pol/UU1R_pol. Products from the RT-PCR were proceeded to nested PCR, using the inner primers UU2L_pol/UU2R_pol. The amplified products were electrophoresed on 1% agarose gel, ethidium-bromide stained, visualized on UV transilluminator. This nested-amplification generated a 1.2 Kbps fragment of the HIV-1 *pol* gene spanning from the 3' end of HIV-1 *gag* to codon 266 of the RT gene. All nested-PCR products from patients in this study are shown in Figure 16. The total amount of amplified DNA was 167.19 ng/μl on average which ranged between 53.24 - 213.74 ng/μl as quantitated by using Picogreen fluorescent dye staining (Invitrogen Oregon, USA). The amplified products were utilized as the template DNA in the OLA.

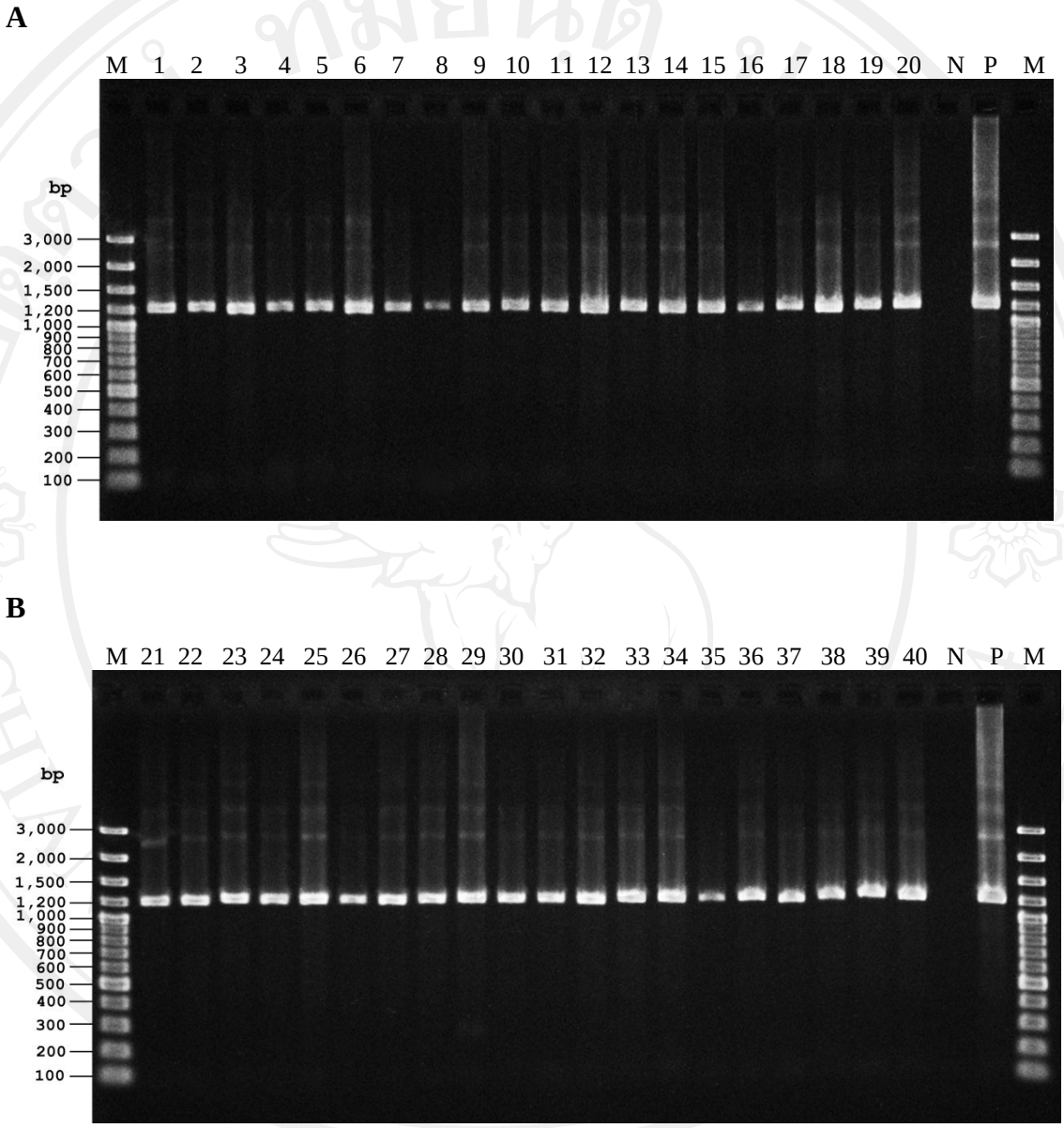

Figure 16 Gel electrophoresis of the nested-PCR products of HIV-1 pol gene in the RT region that served as the template in the OLA. Figure 16A is the gel electrophoresis of the first 20 clinical specimens, Figure 16B is the gel electrophoresis of other 20 clinical specimens. Lane M = 100 bp DNA ladder, Lane N = Negative control, Lane P = Positive control

5.4 Reference plasmids containing wild-type (184M) and mutant variants (184V)

Samples with known M184V genotypes were used to prepare the reference plasmids as the controls for the HIV-1 wild-type (184M) and mutant (184V) detection and optimization of OLA. These plasmids were prepared by cloning of HIV-1 *pol* PCR fragment into the pCR 2.1 TOPO vector as described in the methods section. They were transformed into chemically competent Top 10 cells (*E.coli* competent cells) and were selected by PCR. The result of gel electrophoresis showed the expected band at approximately 1,500 bp by using the outer primers (M13For and M13Rev primers) and showed expected band at approximately 1,200 bp by using and inner primers (UU2L_pol and UU2R_pol primers). They are illustrated as in Figure 17. Afterward, purified plasmid DNA were sequenced and then submitted to geno2pheno software in order to obtain the genotypic information of codon 184 in the RT gene as illustrated in Figures 18 and 19. The nucleotide sequencing results of plasmid DNA showed that HM12, HIV_BaL and CM019 plasmid DNA were HIV-1 184M-wild-type sequences, while CM020 and CM028 plasmid DNA were HIV-1 184V-mutant sequences. These purified plasmids DNA had been confirmed the presence of the wild-type or mutant codon in the HIV-1 *pol* gene were then amplified by PCR and were used as the controls of HIV-1 wild-type and mutant detections by the OLA. All Plasmids for M184V in this study included the wild-type and mutant reference genotypes as shown in Table 6.

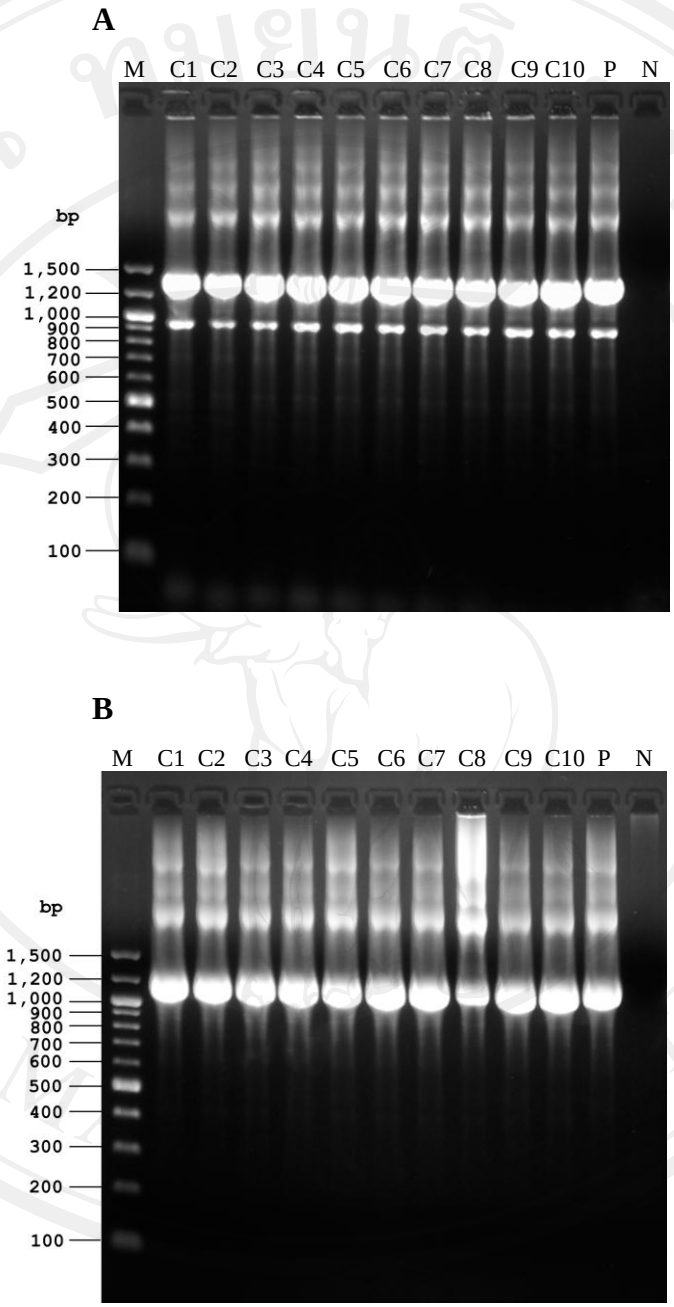

Figure 17 Gel electrophoresis of the amplification of HIV-1 plasmid DNA, cloned 1-10. Figure 17A is gel electrophoresis of the amplified product by using outer primer; M13For and M13Rev, the expected band is approximately 1,500 bp. Figure 17B is gel electrophoresis of the amplified product by using inner primer; UU2L_pol and UU2R_pol, the expected band is approximately 1,200 bp. Lane M = 100 bp DNA ladder, Lane N = Negative control, Lane P = Positive control

Table 6 Nucleotide sequences of HIV-1 M184V wild-type and mutant plasmid controls.

Sample	Results by sequencing	Sequence (5' to 3')	% homology with consensus	Plasmid DNA concentration (ng/ul)
HM12	184M	A GAA AT**G** GTT ATC TAT CAA TAC ATG GAT GAC TTG TAT GTA GGA TC	100	35.8
HIV_BaL	184M	A GAA ATA GT**G** ATC TAT CAA TAC ATG GAT GA**T** TTG TAT GTA GGA TC	96	270.75
CM19	184M	**T** GAA ATG **A**TT ATC **AT**T CAA TAC ATG GAT GAC **C**TG TAT GTA GGA TC	89	133.25
CM020	184V	A GAA AT**G** GTT ATC T**G**T CAA TAC GTG GAT GAC TTG TAT GTA GGA TC	96	305.0
CM028	184V	A GAA AT**G** GTT ATC TAT CAA TAC GTG GAT GAC TTG TAT GTA GGA TC	96	240.5
Consensus	184M	A GAA ATR GTT ATC TAT CAA TAC ATG GAT GAC TTG TAT GTA GGA TC		

Underlined type indicates HIV-1 sequence at 184 codon in reverse transcriptase which was the drug resistance codon tested in the assay. **Boldface type** indicates base changes that no longer complementary to consensus sequence.

geno2pheno

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[Protease | Reverse transcriptase | Subtype | Predicted phenotype | New sequence | PDF Output]

Sequence identifier: Aug 3, 2011

Alignment to reference strain **HXB2**. Position numbering follows Korber *et al.*

Protease

```
ref.   nt: CCTCAGGTCACTCTTTGGCAACGACCCCTCGTCACAATAAAGATAGGGGGGCAACTAAAG 2312
ref.   aa:  P  Q  V  T  L  W  Q  R  P  L  V  T  I  K  I  G  G  Q  L  K    20
query aa:  P  Q  I  T  L  R  Q  R  P  L  V  T  V  K  I  G  G  Q  L  K
query nt: CCTCAAATCACTCTTCGGCAACGACCCCTTGTCACAGTAAAAATAGGAGGACAGTTGAAA

ref.   nt: GAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGAGTTTGCCAGGA 2372
ref.   aa:  E  A  L  L  D  T  G  A  D  D  T  V  L  E  E  M  S  L  P  G    40
query aa:  E  A  L  L  D  T  G  A  D  D  T  V  L  E  D  I  G  L  P  G
query nt: GAAGCGCTATTAGATACAGGAGCAGATGACACAGTATTAGAAGATATAGGTTTGCCAGGG

ref.   nt: AGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTTATCAAAGTAAGACAGTATGAT 2432
ref.   aa:  R  W  K  P  K  M  I  G  G  I  G  G  F  I  K  V  R  Q  Y  D    60
query aa:  K  W  K  P  K  M  I  G  G  I  G  G  F  I  R  V  R  Q  Y  D
query nt: AAATGGAAACCAAAAATGATAGGGGGAATTGGAGGATTTATCAGGGTAAGACAATATGAT

ref.   nt: CAGATACTCATAGAAATCTGTGGACATAAAGCTATAGGTACAGTATTAGTAGGACCTACA 2492
ref.   aa:  Q  I  L  I  E  I  C  G  H  K  A  I  G  T  V  L  V  G  P  T    80
query aa:  Q  I  L  I  E  I  C  G  K  K  A  I  G  T  V  L  V  G  P  T
query nt: CAGATACTTATAGAAATTTGTGGAAAAAAGGCTATAGGTACAGTGTTAGTAGGACCTACA

ref.   nt: CCTGTCAACATAATTGGAAGAAATCTGTTGACTCAGATTGGTTGCACTTTAAATTTT    2549
ref.   aa:  P  V  N  I  I  G  R  N  L  L  T  Q  I  G  C  T  L  N  F      99
query aa:  P  V  N  I  I  G  R  N  M  L  T  Q  I  G  C  L  *  F  P
query nt: CCTGTCAACATAATTGGACGAAATATGTTGACTCAGATTGGTTGTCTTTAATTTCCC
```

bad characters removed:	117	matches in reference sequence:	85.86 %
aligned amino acids:	99	matches in alignment:	85.86 %
insertions / amino acids in total:	0 / 0	deletions / amino acids in total:	0 / 0

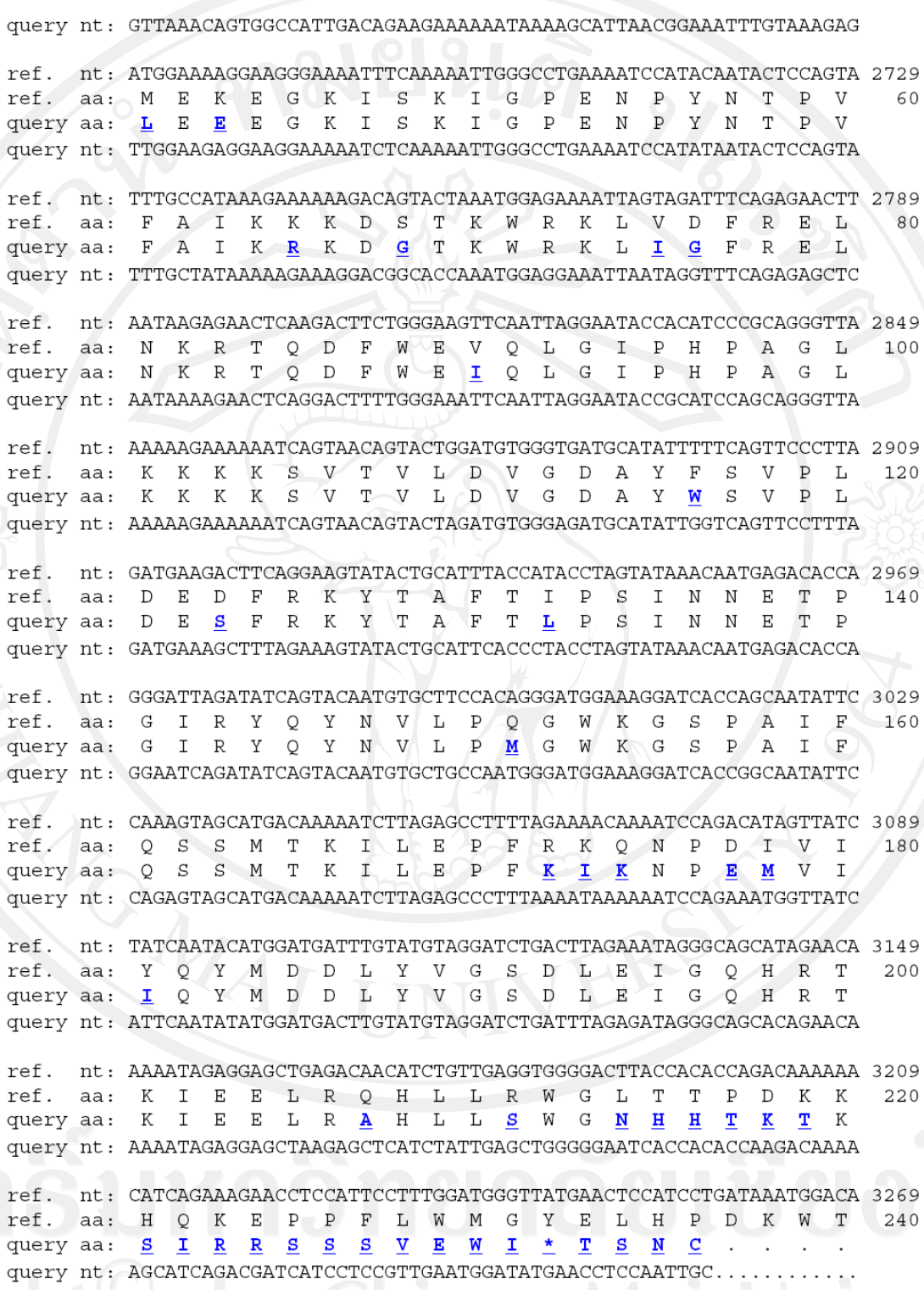
substitutions: V 3 I, W 6 R, I 13 V, E 35 D, M 36 I, S 37 G, R 41 K, K 55 R, H 69 K, L 89 M, T 96 L, L 97 *, N 98 F, F 99 P

Reverse transcriptase

```
ref.   nt: CCCATTAGCCCTATTGAGACTGTACCAGTAAAATTAAAGCCAGGAATGGATGGCCCAAAA 2609
ref.   aa:  P  I  S  P  I  E  T  V  P  V  K  L  K  P  G  M  D  G  P  K    20
query aa:  P  I  S  P  F  D  T  V  P  V  K  L  K  P  G  M  D  G  P  K
query nt: CCAATTAGTCCTTTTGACACTGTACCAGTAAAATTAAAGCCAGGAATGGATGGACCAAAG

ref.   nt: GTTAAACAATGGCCATTGACAGAAGAAAAAATAAAAGCATTAGTAGAAATTTGTACAGAG 2669
ref.   aa:  V  K  Q  W  P  L  T  E  E  K  I  K  A  L  V  E  I  C  T  E    40
query aa:  V  K  Q  W  P  L  T  E  E  K  I  K  A  L  T  E  I  C  K  E
```

Figure 18 An example of sequence alignment of HIV-1 184M-wild-type.



bad characters removed:	117	matches in reference sequence:	34.11 %
aligned amino acids:	236	matches in alignment:	80.93 %

Figure 18 (continue).

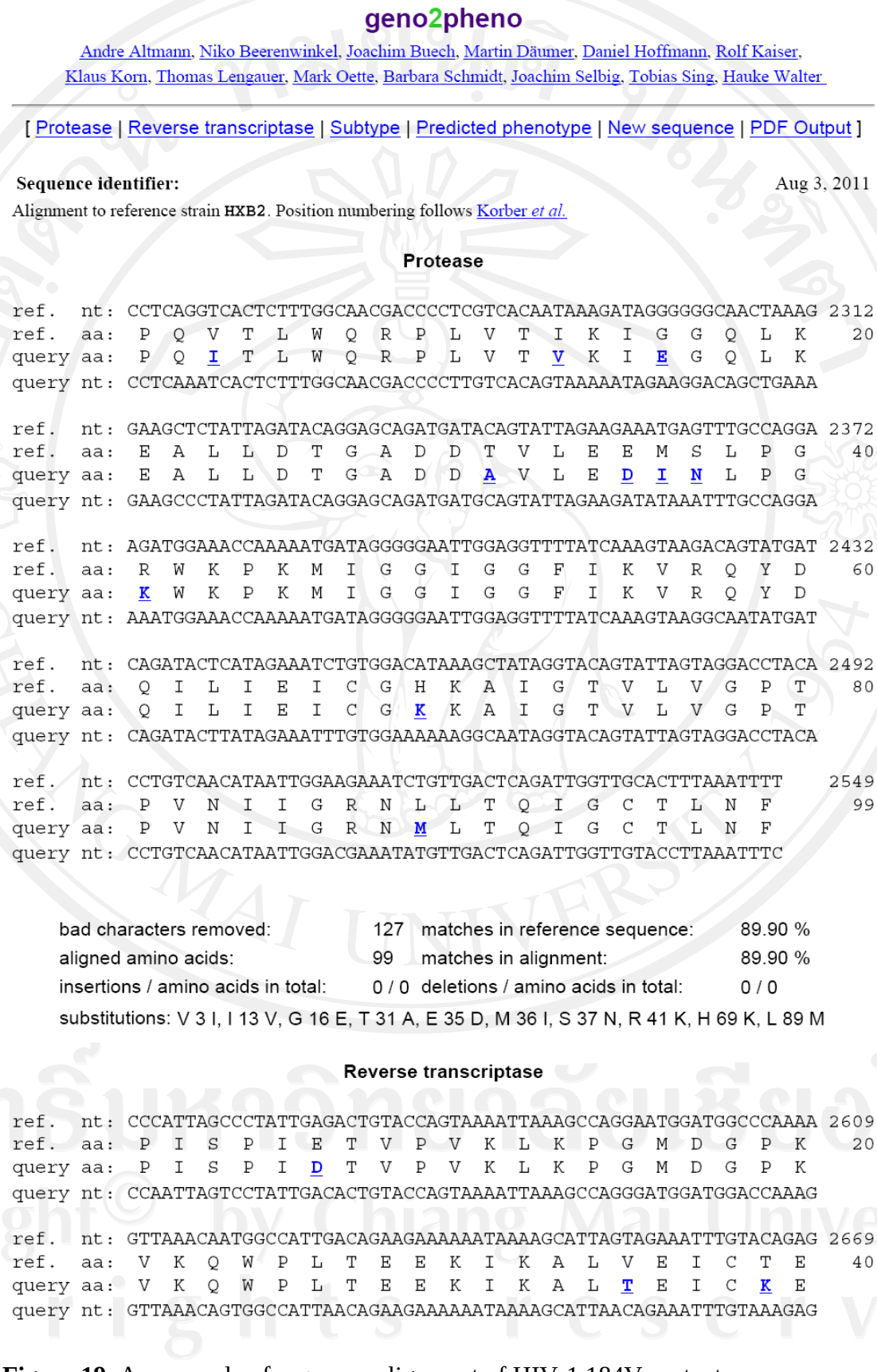
insertions / amino acids in total: 0 / 0 deletions / amino acids in total: 0 / 0

substitutions: I 5 F, E 6 D, V 35 T, T 39 K, M 41 L, K 43 E, K 65 R, S 68 G, V 75 I, D 76 G, V 90 I, F 116 W, D 123 S, I 132 L, Q 151 M, R 172 K, K 173 I, Q 174 K, D 177 E, I 178 M, Y 181 I, Q 207 A, R 211 S, L 214 N, T 215 H, T 216 H, P 217 T, D 218 K, K 219 T, H 221 S, Q 222 I, K 223 R, E 224 R, P 225 S, P 226 S, F 227 S, L 228 V, W 229 E, M 230 W, G 231 I, Y 232 *, E 233 T, L 234 S, H 235 N, P 236 C

Subtype Prediction

Subtype	Probability
01_AE	100.00 %

Figure 18 (continue).



geno2pheno

Andre Altmann, Niko Beerenwinkel, Joachim Buech, Martin Däumer, Daniel Hoffmann, Rolf Kaiser, Klaus Korn, Thomas Lengauer, Mark Oette, Barbara Schmidt, Joachim Selbig, Tobias Sing, Hauke Walter

[Protease | Reverse transcriptase | Subtype | Predicted phenotype | New sequence | PDF Output]

Sequence identifier: Aug 3, 2011

Alignment to reference strain **HXB2**. Position numbering follows Korber *et al.*

Protease

```
ref.   nt: CCTCAGGTCACTCTTTGGCAACGACCCCTCGTCACAATAAAGATAGGGGGGCAACTAAAG 2312
ref.   aa:  P  Q  V  T  L  W  Q  R  P  L  V  T  I  K  I  G  G  Q  L  K    20
query aa:  P  Q  I  T  L  W  Q  R  P  L  V  T  V  K  I  E  G  Q  L  K
query nt: CCTCAAATCACTCTTTGGCAACGACCCCTTGTCACAGTAAAAATAGAAGGACAGCTGAAA

ref.   nt: GAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGAGTTTGCCAGGA 2372
ref.   aa:  E  A  L  L  D  T  G  A  D  D  T  V  L  E  E  M  S  L  P  G    40
query aa:  E  A  L  L  D  T  G  A  D  D  A  V  L  E  D  I  N  L  P  G
query nt: GAAGCCCTATTAGATACAGGAGCAGATGATGCAGTATTAGAAGATATAAATTTGCCAGGA

ref.   nt: AGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTTATCAAAGTAAGACAGTATGAT 2432
ref.   aa:  R  W  K  P  K  M  I  G  G  I  G  G  F  I  K  V  R  Q  Y  D    60
query aa:  K  W  K  P  K  M  I  G  G  I  G  G  F  I  K  V  R  Q  Y  D
query nt: AAATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTTATCAAAGTAAGGCAATATGAT

ref.   nt: CAGATACTCATAGAAATCTGTGGACATAAAGCTATAGGTACAGTATTAGTAGGACCTACA 2492
ref.   aa:  Q  I  L  I  E  I  C  G  H  K  A  I  G  T  V  L  V  G  P  T    80
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ref.   nt: CCTGTCAACATAATTGGAAGAAATCTGTTGACTCAGATTGGTTGCACTTTAAATTTT    2549
ref.   aa:  P  V  N  I  I  G  R  N  L  L  T  Q  I  G  C  T  L  N  F      99
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```

bad characters removed:	127	matches in reference sequence:	89.90 %
aligned amino acids:	99	matches in alignment:	89.90 %
insertions / amino acids in total:	0 / 0	deletions / amino acids in total:	0 / 0

substitutions: V 3 I, I 13 V, G 16 E, T 31 A, E 35 D, M 36 I, S 37 N, R 41 K, H 69 K, L 89 M

Reverse transcriptase

```
ref.   nt: CCCATTAGCCCTATTGAGACTGTACCAGTAAAATTAAAGCCAGGAATGGATGGCCCAAAA 2609
ref.   aa:  P  I  S  P  I  E  T  V  P  V  K  L  K  P  G  M  D  G  P  K    20
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ref.   nt: GTTAAACAATGGCCATTGACAGAAGAAAAAATAAAAGCATTAGTAGAAATTTGTACAGAG 2669
ref.   aa:  V  K  Q  W  P  L  T  E  E  K  I  K  A  L  V  E  I  C  T  E    40
query aa:  V  K  Q  W  P  L  T  E  E  K  I  K  A  L  T  E  I  C  K  E
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```

Figure 19 An example of sequence alignment of HIV-1 184V-mutant.

```
ref.   nt: ATGGAAAAGGAAGGGAAAATTTCAAAAATTGGGCCTGAAAATCCATACAATACTCCAGTA 2729
ref.   aa:  M  E  K  E  G  K  I  S  K  I  G  P  E  N  P  Y  N  T  P  V    60
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query nt: ATGGAAGAGGAAGGAAAAATCTCAAAAATTGGGCCTGAAAATCCATACAATACTCCAGCA

ref.   nt: TTTGCCATAAAGAAAAAAGACAGTACTAAATGGAGAAAATTAGTAGATTTCAGAGAACTT 2789
ref.   aa:  F  A  I  K  K  K  D  S  T  K  W  R  K  L  V  D  F  R  E  L    80
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query nt: TTTGTTATAAAGAAAAAGGACGGCATCAAATGGAGGAAATTAACAGATTTCAGAGAACTC

ref.   nt: AATAAGAGAACTCAAGACTTCTGGGAAGTTCAATTAGGAATACCACATCCCGCAGGGTTA 2849
ref.   aa:  N  K  R  T  Q  D  F  W  E  V  Q  L  G  I  P  H  P  A  G  L   100
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ref.   nt: GATGAAGACTTCAGGAAGTATACTGCATTTACCATACCTAGTATAAACAATGAGACACCA 2969
ref.   aa:  D  E  D  F  R  K  Y  T  A  F  T  I  P  S  I  N  N  E  T  P   140
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ref.   nt: GGGATTAGATATCAGTACAATGTGCTTCCACAGGGATGGAAAGGATCACCAGCAATATTC 3029
ref.   aa:  G  I  R  Y  Q  Y  N  V  L  P  Q  G  W  K  G  S  P  A  I  F   160
query aa:  G  I  R  Y  Q  Y  N  V  L  P  Q  G  W  K  G  S  P  A  I  F
query nt: GGAATCAGATATCAGTACAATGTGCTGCCACAGGGATGGAAAGGGTCACCAGCAATATTT

ref.   nt: CAAAGTAGCATGACAAAAATCTTAGAGCCTTTTAGAAAACAAAATCCAGACATAGTTATC 3089
ref.   aa:  Q  S  S  M  T  K  I  L  E  P  F  R  K  Q  N  P  D  I  V  I   180
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ref.   nt: TATCAATACATGGATGATTTGTATGTAGGATCTGACTTAGAAATAGGGCAGCATAGAACA 3149
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query nt: TATCAATACGTGGATGACTTGTATGTAGCATCTGACTTAGAAATAGGGCAGCACGGGAGC

ref.   nt: AAAATAGAGGAGCTGAGACAACATCTGTTGAGGTGGGGACTTACCACACCAGACAAAAAA 3209
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query aa:  K  I  R  A  S  N  Q  .  .  .  .  .  .  .  .  .  .  .  .  .  .
query nt: AAAATCCGAGCTTCGAACCAG.....................................
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bad characters removed:	127	matches in reference sequence:	32.68 %
aligned amino acids:	207	matches in alignment:	70.38 %

Figure 19 (continue).

insertions / amino acids in total: 1 / 53 deletions / amino acids in total:

substitutions: E 6 D, V 35 T, T 39 K, K 43 E, V 60 A, A 62 V, S 68 G, T 69 I, V 75 T, K 101 H, D 123 S, M 164 T, K 173 I, Q 174 K, D 177 E, I 178 M, M 184 V, G 190 A, R 199 G, T 200 S, E 203 R, E 204 A, L 205 S, R 206 N

Subtype Prediction

Subtype	Probability
01_AE	100.00 %

Figure 19 (continue).

5.5 Optimization of OLA conditions

In the step of OLA optimization, three important components; the thermostable DNA ligase enzyme, the probes (wild-type detector-, mutant detector-probes, and common probes) and the templates in the OLA reaction were verified in order to obtain the optimal condition in differentiating the wild-type (184M) (amplified PCR products of HM-12 plasmid control) and mutant (184V) variants (amplified PCR products of CM020 and CM028 plasmid control). The titration curves were plotted, and the amount of which that reached plateau of the titration curve was considered optimal. The results of OLA optimization by using the mutant probe (MT_184V probe) system are shown in Figures 20, 22 and 24. The results by using the modified mutant probe (Mod_MT184V probe) system are show in Figures 21, 23 and 25.

From the results of DNA ligase enzyme optimization, the different concentration of enzyme at 0.04, 0.08, 0.17, 0.34, 0.68 U were used. The optimal enzyme concentration for OLA was 0.34 U. The results of DNA ligase enzyme titration are shown in Figure 20 for MT_184V probe system. Figure 21 illustrated the results of enzyme titration for Mod_MT184V probe system.

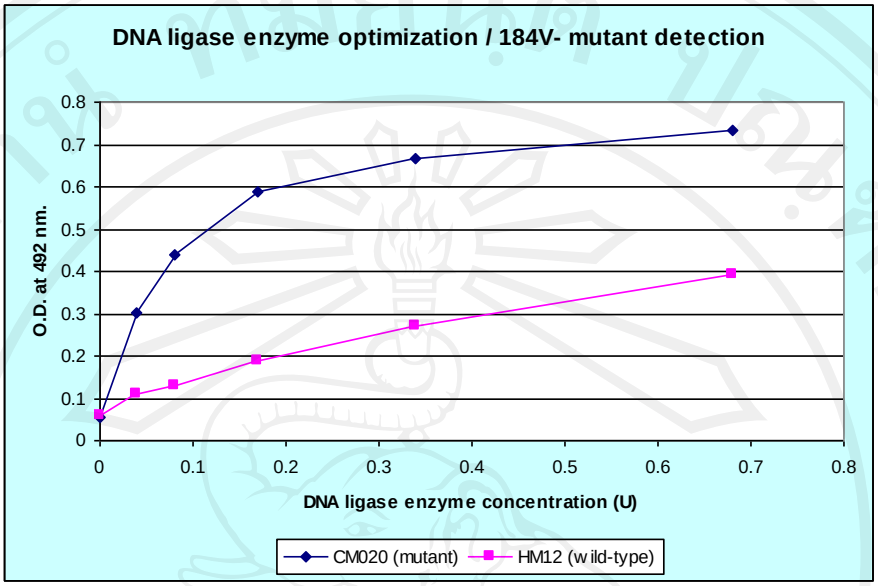
For the probes optimization, the different amount of probes were 0.06, 0.12, 0.36, 1.08, 2.16 pmol. The probes concentration at 0.36 pmol was the optimal concentration. The results of probes titration is shown in Figure 22 for MT_184V probe system, whereas, Figure 23 shown the results of probe titration for Mod_MT184V probe system.

For the templates titration, the different volumes of template at 0, 2.5, 5, 10, 0.05, 0.1, 1 µl were used. The optimal template volume for OLA was at least 0.1 µl.

The result of template volume titration is shown in Figure 24 for MT_184V probe system. Beside, for template concentration titration, the different amount of reference plasmids, at 0, 2.5, 5, 10, 25, 50, 100 ng were used. The minimal template at 50 ng was optimally detected and discriminated by OLA. Figure 25 shows the results of template titration for Mod_MT184V probe system.

According to the DNA concentration in the PCR products ranged 53.24 - 213.74 ng/µl, therefore, using 1 µl of amplified DNA products was sufficient for template in the OLA reaction. From the overall results of OLA optimization, due to using the MT_184V probe system counterpart encountered a high background of OD_{492} measurement on the wild-type templates (see Figures 20A, 22A and 24A). Whereas, the modified mutant probe (Mod_MT184V probe) system could discriminated both wild-type and mutant template exactly distinguished. Therefore, the Mod_MT184V probe system was mainly used to do OLA in the study.

A



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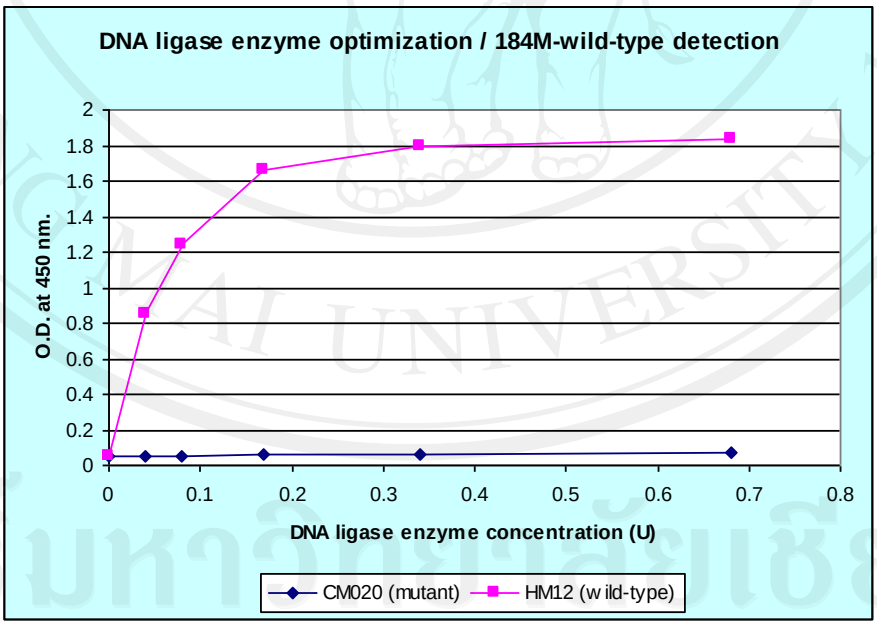
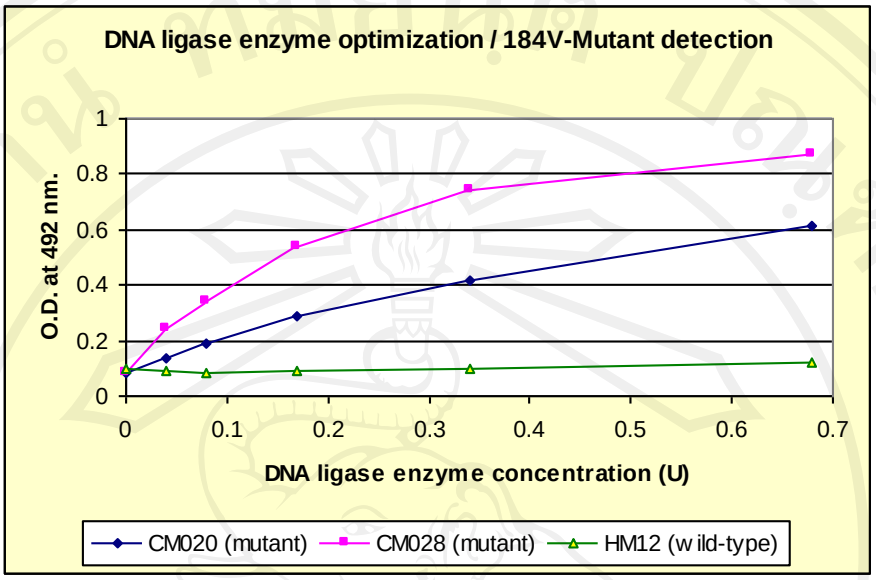


Figure 20 Optimization of DNA ligase enzyme concentration for the detection of HIV-1 M184V mutation by OLA with MT_184V probe system. Figure 20A Mutant detection. Figure 20B Wild-type detection.

A



B

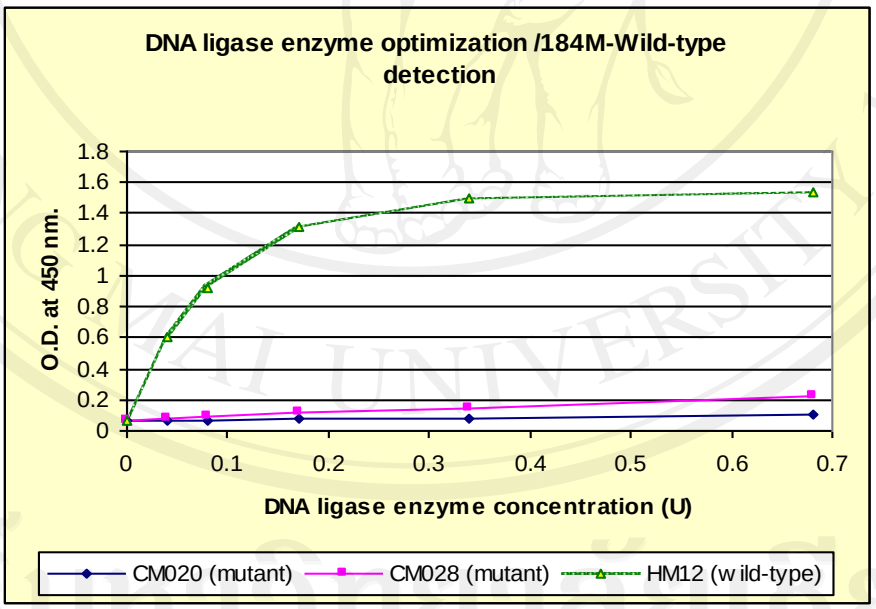
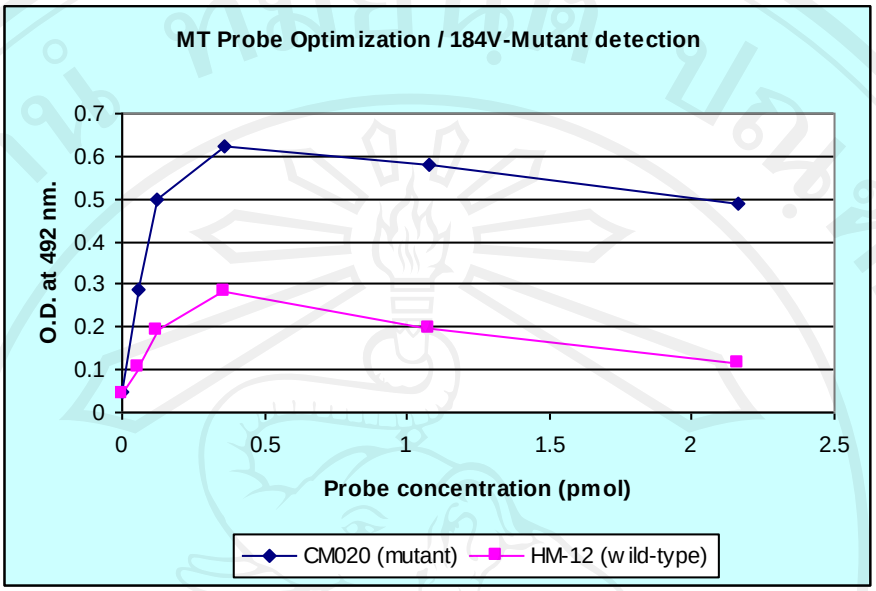


Figure 21 DNA ligase enzyme concentration optimization for HIV-1 M184V mutation detection by OLA with Mod_MT184V probe system. Figure 21A Mutant detection. Figure 21B Wild-type detection.

A



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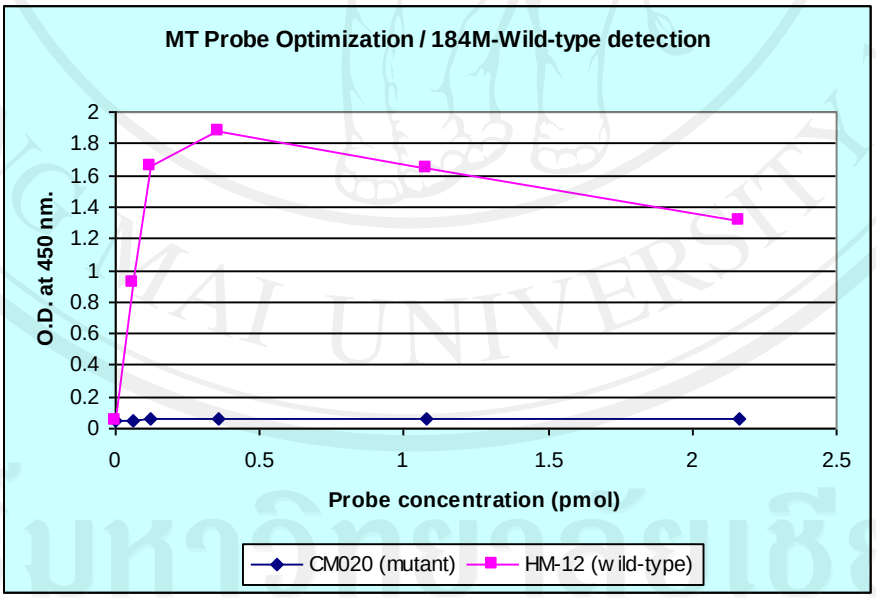
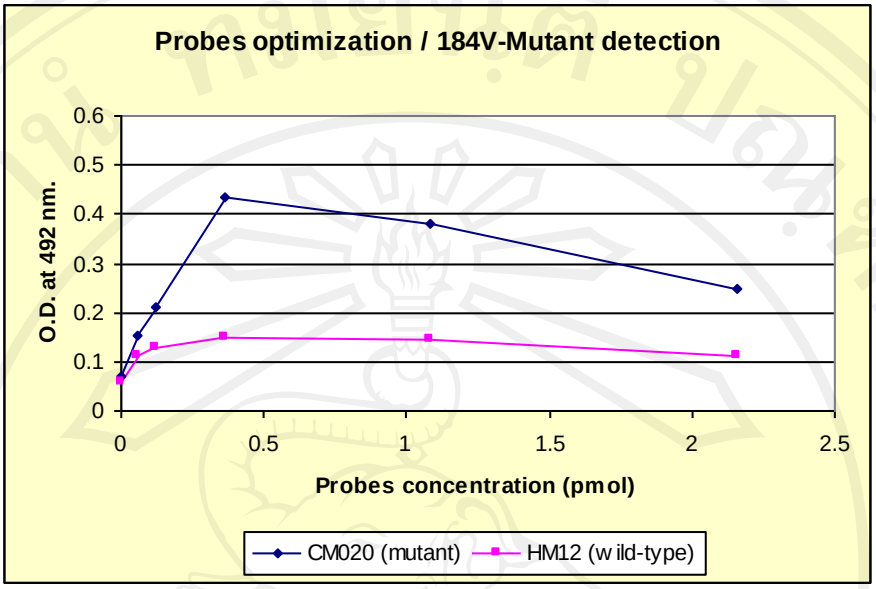


Figure 22 Optimization of probes concentration for the detection of HIV-1 M184V mutation by OLA with MT_184V probe system. Figure 22A Mutant detection. Figure 22B Wild-type detection.

A



B

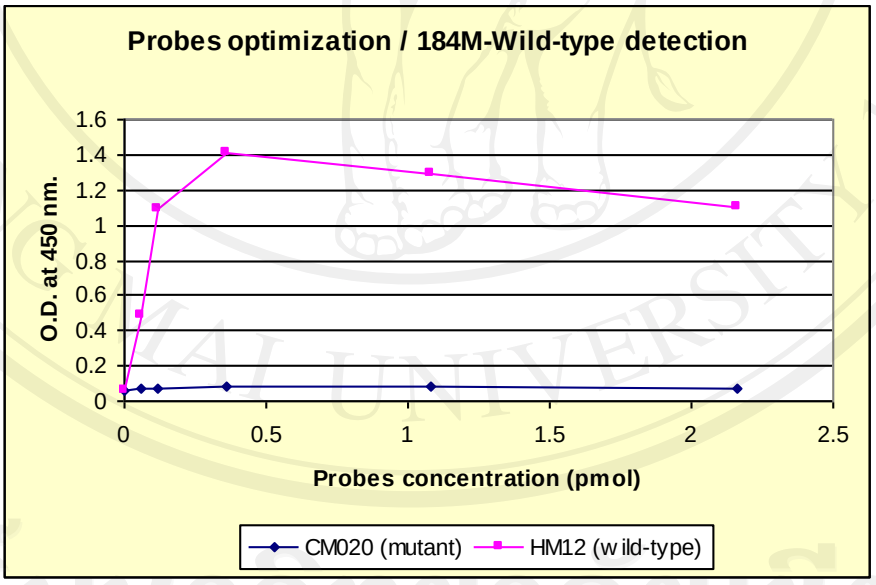
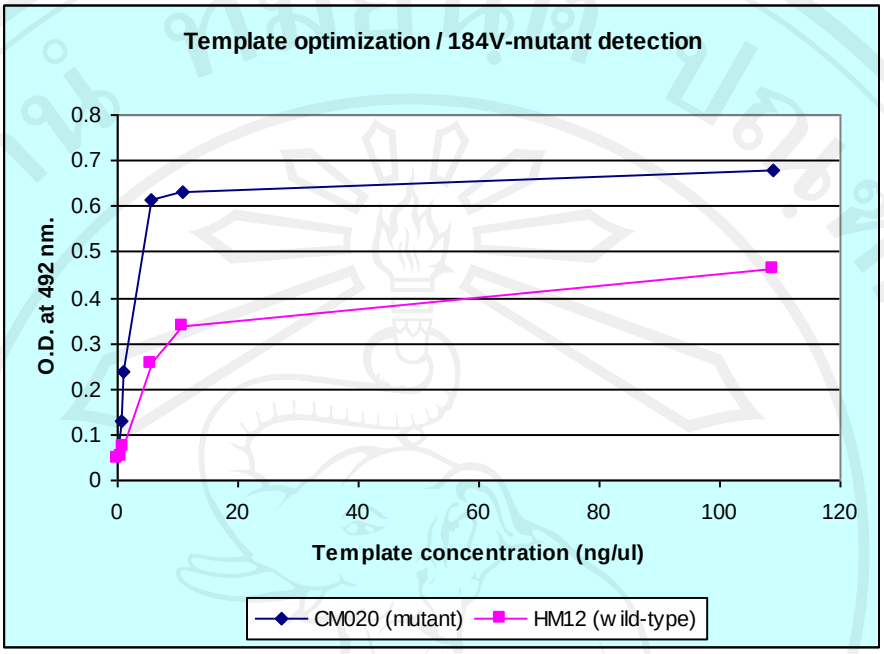


Figure 23 Probes concentration optimization for HIV-1 M184V mutation detection by OLA with Mod_MT184V probe system. Figure 23A Mutant detection. Figure 23B Wild-type detection.

A



B

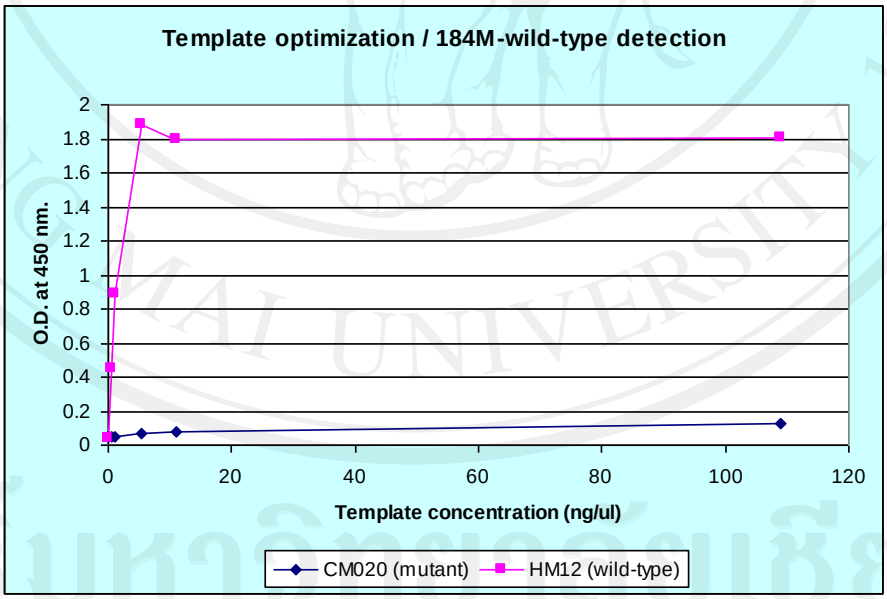
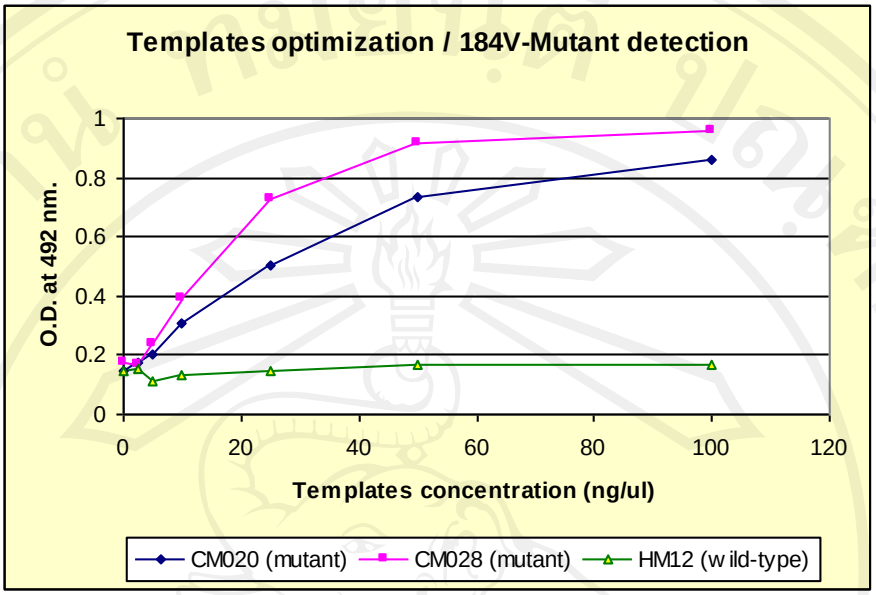


Figure 24 Templates optimization for HIV-1 M184V mutation detection by OLA with MT_184V probe system. Figure 24A Mutant detection. Figure 24B Wild-type detection.

A



B

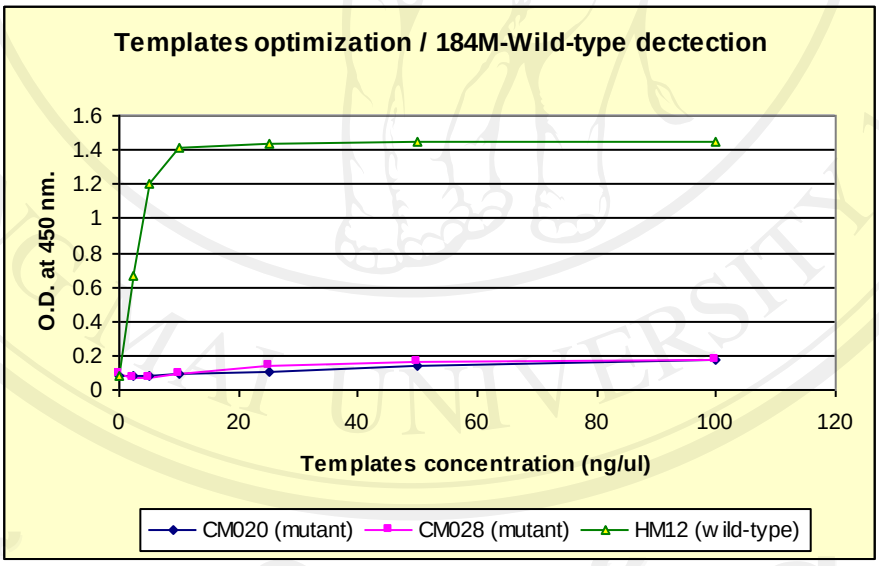


Figure 25 Templates optimization for HIV-1 M184V mutation detection by OLA with Mod_MT184V probe system. Figure 25A Mutant detection. Figure 25B Wild-type detection.

5.6 The lower limit of detection of the mutant detection by OLA

The lower limit of detection of mutant (M184V) variants detection were determined using the mixtures of amplified PCR products of wild-type and mutant plasmids control in parallel with 100% wild-type (184M) and 100% mutant (184V). The amplified PCR products of HM12 wild-type plasmid control were mixed with increasing amount of 1%, 3%, 5%, 10%, 20%, 30%, 40%, and 50% of the amplified PCR products of CM020 and CM028 mutant plasmid control. As shown in Figure 26, the mixture containing 3% mutant of CM28A clone gave the OD_{492} signal above cut-off and that from CM020 mutant clone gave the OD_{492} at marginal to the cut-off. The mixture containing 5% of either one was more reliable for positive detection that the OD_{492} measurements were higher than the cut-off level. Therefore, the overall results showed that the mutant variants present at 3%-5% in the PCR products could be detected by OLA.

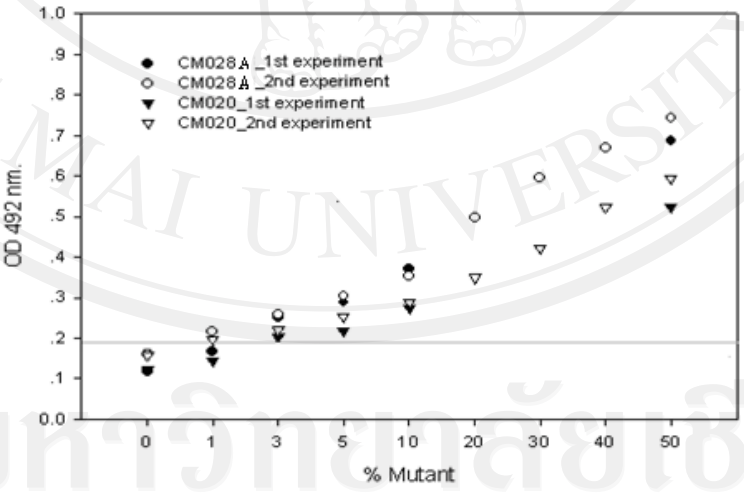


Figure 26 The low limit detection of OLA determination using mixture of mutant clones, CM028A or CM020, at different proportion in the background of wild-type clone (HM-12). The indicator line is the level of Mean + 3 SD of the background (0% mutant).

After lower limit of mutant detection by OLA was determined, further experiments were tested to ensure the reliability of OLA to detect the mutant variants present at 3%-5% in the PCR products. The mixture of the amplified PCR products of wild-type plasmid control with increasing amount of 1%, 3% and 5% of the amplified PCR product of mutant plasmid control were tested by OLA for 5-10 replicated reaction. As shown in Table 7, the mutant CM020 presented at 3%-5% and mutant CM028 as low as 1% in the PCR products could be reliably detected by OLA ($p<0.005$).

Table 7 Detection of MT in the mixtures of WT and MT at different concentration

		CM020			CM028		
Blank	**0% MT**	**1% MT**	**3% MT**	**5% MT**	**1% MT**	**3% MT**	**5% MT**
0.058	0.118	0.121	0.132	0.13	0.133	0.193	0.232
H2O	0.116	0.121	0.122	0.142	0.126	0.174	0.214
0.063	0.114	0.145	0.141	0.154	0.108	0.171	0.243
	0.11	0.125	0.137	0.141	0.137	0.172	0.223
	0.114	0.104	0.122	0.14	0.134	0.17	0.236
	0.113	0.112	0.122		0.138	0.166	
	0.126	0.112	0.125		0.147	0.167	
	0.113	0.118	0.143		0.147	0.172	
	0.106	0.114	0.14		0.14	0.168	
	0.126	0.122	0.122		0.134	0.172	
Sum	1.156	1.194	1.306	0.707	1.344	1.725	1.148
Mean	**0.116**	**0.119**	**0.131**	**0.141**	**0.134**	**0.173**	**0.230**
SD.	0.006	0.011	0.009	0.009	0.011	0.008	0.011
Mean+3SD	**0.135**	**0.152**	**0.157**	**0.167**	**0.168**	**0.195**	**0.264**
P VALUE		**0.460**	**0.0004**	**0.000**	**0.0003**	**0.0000**	**0.0000**

WT = wild-type genotype, MT=mutant genotype

5.7 Performance of OLA on small set of plasmid controls

The performance of M184V detection by OLA in a small set of plasmid controls was evaluated. These plasmids representing wild-type (HM-12, BaL, CM019) and mutant (CM20, CM28) genotypes at codon 184 in the RT-gene (see Table 6). The amplified PCR products of these reference plasmid controls were used as the template for OLA to detect HIV-1 M184V mutation by using the OLA conditions obtained from the OLA optimization. The OLA result of M184V mutation detection represent 100% concordance with standard nucleotide sequencing results. These results are shown in Table 8.

Table 8 Results of the performance evaluation of OLA and standard sequence analysis for the M184V mutation detection of known genotype plasmid DNA.

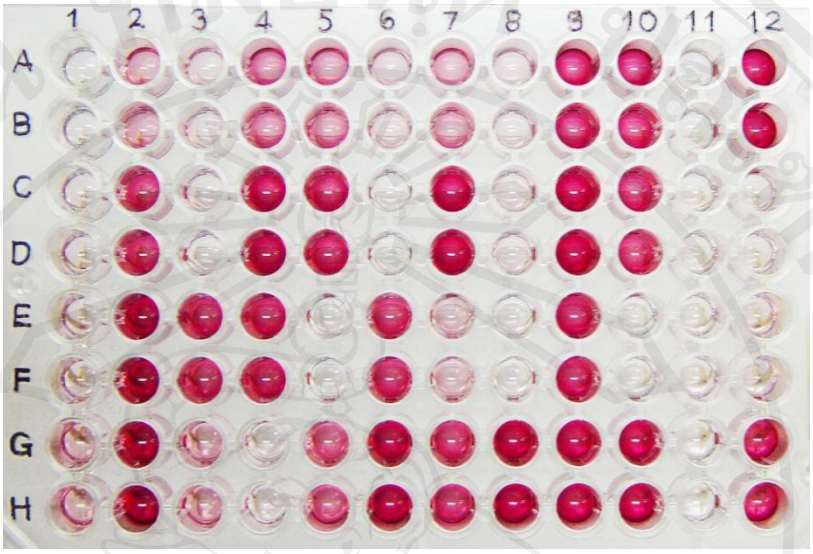
Plasmid No.	Subtype	Standard sequencing results	OLA results
CM019	CRF01_AE	184M - wild-type	wild-type
HM12	CRF01_AE	184M - wild-type	wild-type
BAL	B	184M - wild-type	wild-type
CM020	CRF01_AE	184V-mutant	mutant
CM028	CRF01_AE	184V-mutant	mutant

5.8 Performance of OLA on clinical specimen testing

The archival plasma specimens from HIV-1 infected patients who fail treatment (N = 40) were served as clinical specimens for this purpose. These specimens have been tested for the HIV-1 drug resistance by the standard nucleotide sequencing using the in-house method at the HIV-1 drug resistance laboratory which was a gold standard method (Praparattanapan et al., 2011). The amplified DNA products of these specimens were used as template for the evaluation of performance of the OLA in detecting HIV-1 M184V drug resistance mutation and all samples were tested in duplicate. The OLA conditions obtained from the OLA optimization were used. The interpretation of OLA, the O.D. value above 0.2 was considered positive for mutant and wild-type detections.

The results of M184V detection by OLA on these specimens is shown in Figure 27A (mutant detection) and Figure 27B (wild-type detection). The results of the OLA showed that among 40 clinical specimens, 23 samples were 184V-mutant genotype, 9 samples were 184M-wild-type genotype, 1 sample (2.5%) was the mix of wild-type and 7 samples were indeterminate result (Table 9). Whereas, the results of samples which were identified by the standard sequencing genotype method shown that 30 samples (75.0%) were exclusively 184V-mutant genotype, 7 samples (17.5%) were 184M-wild-type, 1 sample (2.5%) was the mix of wild-type and 184V-mutant and 2 samples (5%) were exclusively 184I-mutant. The results of OLA and standard sequencing genotype method were shown in Table 10.

A



B

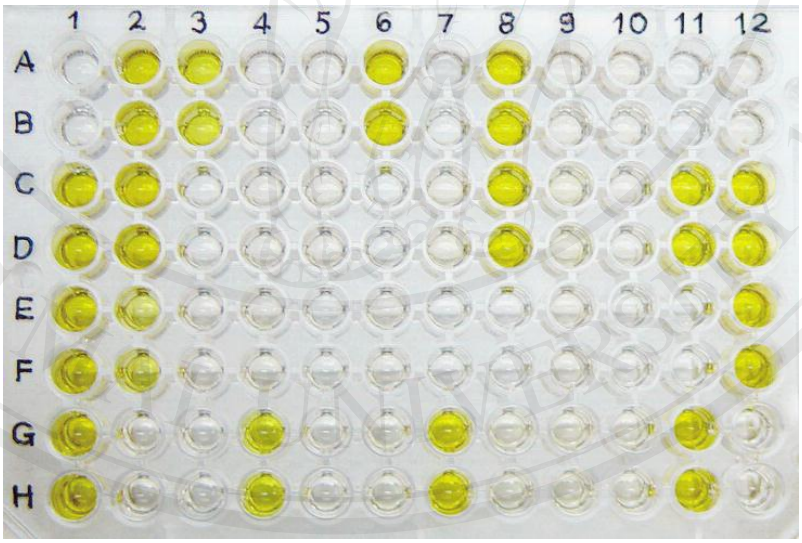


Figure 27 The 40 clinical specimen results of HIV-1 M184V mutation detection by OLA with Mod_MT184V probe system. Figure 27A is shown samples with 184V-mutant genotypes produce a magenta color after addition of alkaline phosphatase substrate and amplifier, while negative samples remain colorless or slightly clear. Figure 27B is shown samples with 184M-wild-type genotypes produce a yellow color after addition H_2SO_4 to stop reaction, while the negative samples remain clear.

Table 9 Interpretation of the detection of M184V mutation of HIV-1 by OLA in clinical specimens (duplicated of S1-S40).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	5% MT C	S1 WT	S5 MT	S9 MT	S13 WT	S17 MT	S21 WT	S25 MT	S29 MT	S37 IND	S33 MT
B	H2O C	5% MT C	S1 WT	S5 MT	S9 MT	S13 WT	S17 MT	S21 WT	S25 MT	S29 MT	S37 IND	S33 MT
C	0% MT C	20% MT C	S2 IND	S6 MT	S10 MT	S14 IND	S18 MT	S22 WT	S26 MT	S30 MT	S38 WT	S34 WT
D	0% MT C	20% MT C	S2 IND	S6 MT	S10 MT	S14 IND	S18 MT	S22 WT	S26 MT	S30 MT	S38 WT	S34 WT
E	1% MT C	95% MT C	S3 MT	S7 MT	S11 IND	S15 MT	S19 MT	S23 IND	S27 MT	S31 IND	S39 IND	S35 WT
F	1% MT C	95% MT C	S3 MT	S7 MT	S11 IND	S15 MT	S19 MT	S23 IND	S27 MT	S31 IND	S39 IND	S35 WT
G	3% MT C	100% MT C	S4 MT	S8 WT	S12 MT	S16 MT	S20 *MIX*	S24 MT	S28 MT	S32 MT	S40 WT	S36 MT
H	3% MT C	100% MT C	S4 MT	S8 WT	S12 MT	S16 MT	S20 *MIX*	S24 MT	S28 MT	S32 MT	S40 WT	S36 MT

C= control, MT=184V-mutant genotype, WT=184M-wild-type genotype, *MIX*= mixture of WT and MT viruses, IND= Indeterminate = neither WT nor MT.

Table 10 Comparison of OLA and standard sequence analysis for the detection of M184V mutation.

Total specimens	**Number of samples with indicated result**							
	OLA				**Standard sequencing**			
	WT	**MT**	**Mix**	**Indeterminate**	**WT**	**MT**	**Mix**	**Other**
40	9	23	1	7	7	30	1	2

WT= 184M-wild-type, MT=184V- mutant, Mix=mixture of WT and MT viruses, Other= other mutation apart from M184V, Indeterminate = neither WT nor MT

From the results of OLA for HIV-1 M184V mutation detection, the comparison of OLA with standard sequence analysis gave a concordance of 77.5% for the M184V mutation detection. The concordance was defined as the same results were obtained by both OLA and sequence analysis. The comparison results of OLA with in-house sequence analysis for M184V mutation is shown in Table 11.

Table 11 Evaluation of OLA with standard sequence analysis for the detection of M184V mutation.

Interpretation	Result		No. of samples	%
	Standard sequencing	OLA		
Concordance	184M-WT 184V-MT Mix (MT & WT)	184M-WT 184V-MT Mix (MT & WT)	7 23 1	77.5 %
Alternate mutation	M184I	184M-WT	2	5 %
Indeterminate result	184M-WT	Indeterminate	0	0 %
	184V-MT	Indeterminate	7	17.5 %
Total			40	100 %

184M-WT; 184M-wild-type genotype (M = methionine, GTG), 184V-MT; 184V-mutant genotype (V = valine = GTG), Mix = mixture of WT and MT virus, Indeterminate = neither WT nor MT

Among 40 clinical specimens identified by the in-house HIV-1 genotypic drug resistant assay, because our current OLA was not designed for 184I detection, therefore, the 2 samples with 184I were removed from the analysis. Excluding the 2 samples with 184I variants, the sensitivity of 100% (8 of 8) and 77.4% (24 of 31) for the wild-type and 184V-mutant detection, respectively, were achieved by the OLA. All of wild-type negative samples (30/30) and all 184V-mutant negative samples (7/7) by sequencing were also negative by OLA, giving a 100% specificity for the wild-

type and 100% specificity for the 184V-mutant detection. Combining the wild-type and 184V-mutant detections, the overall sensitivity and specificity of HIV-1 M184V detections by OLA were 81.6% (31 of 38) and 100%, respectively. [Sensitivity = (number of true positive samples / number of true positive samples + number of false negative samples) x 100]. Sensitivity was defined as the ability of the assay to correctly identify all true positive samples in the test (wild-type and/or mutant).

Five percents (2 of 40) of the samples were 184I variants (ID# S8, S38), which were not the target of our designed probes, and thus were not detected by OLA. However, the OLA was able to recognize the presence of wild-type strains in these samples which were missed by in-house sequence analysis.

Seven clinical specimens with indeterminate OLA results (ID# S2, S11, S14, S23, S31, S37, S39) were further analyzed. These specimens were retested with the MT-184V probe system and the nucleotide sequences within the OLA probes binding these specimens region were evaluated by standard DNA sequencing.

From indeterminate specimens, five of seven specimens turned positive for mutant genotypes by OLA when retesting using the MT-184V probe. Whereas, other two specimens (2/40, 5.0%) (ID# S2, S11) also provided indeterminate results even using probes set that include the MT-184V mutant detector probe.

All of the results were recalculated; the comparison of OLA with standard sequence analysis gave a concordance increased to 90.0% (36/40). Furthermore, the overall sensitivity of OLA to detect 184V-mutant was 93.5% (29/31). The percentage of indeterminate result was decreased from 17.5% (7/40) to 5% (2/40). The results show in Table 12.

Table 12 The overall results of comparison of OLA with standard sequence analysis for the detection of M184V mutation.

Interpretation	Result		No. of samples	%
	Standard sequencing	OLA		
Concordance	184M-WT 184V-MT Mix (MT & WT)	184M-WT 184V-MT Mix (MT & WT)	7 28 1	90.0 %
Alternate mutation	M184I	184M-WT	2	5 %
Indeterminate result	184M-WT	Indeterminate	0	0 %
	184V-MT	Indeterminate	2	5 %
Total			40	100 %

184M-WT; 184M-wild-type genotype (M = methionine, GTG), 184V-MT; 184V-mutant genotype (V = valine = GTG), Mix = mixture of WT and MT virus, Indeterminate = neither WT nor MT

Furthermore, the nucleotide sequences of seven indeterminate clinical specimens were evaluated by standard DNA sequencing by using our system to determine the nucleotide sequences of sample. Prior to directly sequencing, residual PCR primers and dNTPs were removed from the amplified PCR products of these clinical specimens by using the PCR clean-up, NucleoSpin® Extract II kit (MACHEREY-NAGEL, Germany). Cleaned PCR products were subjected to directly sequencing using forward primer UU2L-pol, reverse primer UU2R_pol and HXB2_2395 primer. All cleaned PCR products were sequenced with fluorescence-labeled dideoxy chain terminators (Big dye version 3.1, Applied Biosystems) on the ABI 3130 Genetic Analyzer (Applied Biosystems/ HITACHI). The multiple nucleotide sequence alignments between the samples and reference serotypes were obtained by using the computer programs. The results of DNA sequencing were shown in Table 13.

From the evaluation of nucleotide sequencing of 7 samples with indeterminate results by OLA, as expected, the nucleotide polymorphisms within the probes binding regions were abundant in these HIV-1 genomes. Importantly, 4 samples had additional changes around the ligation site (Table 13). The presence of several polymorphisms located in the sample's sequence, resulting in decreased complementary to the probe and may explain the indeterminate results by OLA.

Table 13 The nucleotide sequencing results of the specimens which had discordance results of HIV-1 M184V mutation between OLA and standard HIV-1 genotypic drug resistant assay.

Sample No.	Results by sequencing	Sequence (5' to 3')	% homology with specific probes
S2	M184V	A GAA ATA GTT ATC TA**C** CA**G** T**AT** G*TG GAT GAC TTG TAT GTA GGA TC	91
S11	M184V	A GAA ATA G**A**T ATC TAT CAA T**AT** G*TG GAT GA**T** TTG **CT**T GTA GGA TC	87
S14	M184V	A GA**C** ATA GTT AT**A** TAT CA**G** T**A**C G*TG GAT GA**T** TTG TAT GTA G**C**A TC	87
S23	M184V	A GA**C** ATA GTT ATC TA**G** CAA T**A**C G*TG GAT GA**T** **C**T**A** TAT GT**T** GGA TC	84
S31	M184V	A GAA ATA GTT ATC T**G**T CAA T**AT** G*TG GAT GAC TTG TAT GTA GGA TC	93
S37	M184V	A GAA ATG **A**TT ATC T**GC** CA**G** T**A**C G*TG GA**C** GA**T** TTG TAT GTA GGA TC	84
S39	M184V	A GAA ATG GTT ATC TAT CAA T**AT** G*TG GAT GAC TTG TAT GTA **AG**C TC	91
Consensus		A GAA ATR GTT ATC TAT CAA TAC ATG GAT GAC TTG TAT GTA GGA TC	
WT_184 Probe		*Dig* – A GAA ATR GTT ATC TAT CAA TAC **A**	
Mod_MT184V Probe		*Flu*- A GAA ATR GTT ATC TAT CAA TCC **G**	
Common_184		*Phosphate*- **TG** GAT GAC TTG TAT GTA GGA TC - *bio*	

Boldface type indicates base changes that no longer complementary to the ligation oligonucleotides included in this study. The drug resistance codon tested in the assay is underlined. An asterisk indicates the ligation site.

WT = 184M-wild-type, MT = 184V-mutant, Common = common probe, *Dig* = Digoxigenin, *Flu* = Fluorescein, *bio* = Biotin